



**Catarina Correia de
Lemos Malheiro**

**Efeitos do biochar na qualidade e remediação de
solos**

Biochar effects on the soil's quality and remediation

DECLARAÇÃO

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**Efeitos do biochar na qualidade e remediação de
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Biochar effects on the soil quality and remediation

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Toxicologia e Ecotoxicologia, realizada sob a orientação científica da Doutora Susana Patrícia Mendes Loureiro, Investigadora Auxiliar do Departamento de Biologia e do CESAM - Centro de Estudos do Ambiente e do Mar da Universidade de Aveiro, e co-orientação da Doutora Ana Catarina Bastos, Investigadora de Pós-Doutoramento do Departamento de Biologia e do CESAM da Universidade de Aveiro.

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palavras-chave

Ecotoxicologia, solos, contaminação, pesticidas, biochar, remediação, *Folsomia candida*, *Brassica rapa*.

resumo

A contaminação dos solos é um problema mundial que necessita de uma resolução. Várias técnicas foram e têm vindo a ser desenvolvidas para verificar a sua eficácia em remover contaminantes orgânicos e inorgânicos dos solos. O biochar é um material carbonáceo que, além de ser um produto de reestruturação de solos, pode imobilizar compostos químicos devido à sua grande área de superfície específica reativa, tornando-os não disponíveis para a biota do solo. Assim, o objetivo deste estudo foi testar a capacidade do biochar em imobilizar dimetoato em solos agrícolas, e, deste modo, diminuir a sua toxicidade para os organismos do solo. Para testar esta hipótese, duas taxas de biochar – 2.5% e 5% (m/m) – e dois organismos modelo – o colêmbolo *Folsomia candida* e a planta *Brassica rapa* – foram escolhidos para estudar a imobilização do pesticida pelo biochar através da avaliação das alterações na toxicidade do dimetoato aquando da inclusão do biochar no solo. Como complemento, análises químicas foram, também, realizadas ao solo e à água dos poros do solo para averiguar se a concentração química diminuiu.

No teste de reprodução com colêmbolos, a produção de juvenis e a taxa de sobrevivência foram afetados positivamente com o tratamento do biochar, independentemente da sua percentagem no solo. Em relação ao teste com as plantas, parâmetros como o comprimento e o peso fresco das partes aéreas destas foram, também, afetadas positivamente com a adição do biochar; contudo, a sua influência foi menos eficiente porque houve uma curva dose-resposta para o pesticida. Com estes resultados, conclui-se que o biochar pode diminuir os efeitos induzidos pelo dimetoato, ao diminuir a sua biodisponibilidade para a fauna e flora do solo.

keywords

Ecotoxicology, soils, contamination, pesticides, biochar, remediation, *Folsomia candida*, *Brassica rapa*.

Abstract

Soil contamination is a worldwide problem urging for resolution. Several techniques are being developed and upgraded to see their efficacy in removing organic and inorganic contaminants from soils. Biochar is a carbonaceous material that, aside from being a soil amendment, can immobilize chemical compounds due to a large and reactive specific surface area, potentially turning them unavailable for the soil biota. Therefore, the aim of this study was to test the biochar's capacity to immobilize dimethoate in agricultural soils and, therefore, decrease the toxicity to soil organisms. To test this hypothesis, two biochar rates – 2.5% and 5% (w/w) - and two standardized organisms - the collembolan *Folsomia candida* and the plant *Brassica rapa* - were chosen to assess pesticide immobilization by biochar by evaluating changes in dimethoate toxicity upon soil amendment. As a complement, chemical analyses were also performed on the soil and on the soil's pore water to check if the chemical concentration decreased.

In the reproduction test with collembolans, the offspring production and the survival rate were affected positively with the biochar treatment, independent of the percentage of biochar in soil amendment. For the germination test, endpoints such as length and fresh weight of the aerial part of the plants were also affected positively with biochar addition; however, biochar's influence was less efficient because there was still a dose-response curve for dimethoate observed. With these results, we can conclude that biochar can alleviate dimethoate pollution, by decreasing the bioavailability to soil fauna and flora.

Index

List of figures	i
List of tables	iii
List of abbreviations	iv
CHAPTER ONE: General Introduction	1
1. General Introduction	3
1.1. Soil contamination	3
1.2. Biochar	5
1.2.1. Introducing biochar: a brief historical record	5
1.2.2. Biochar production	7
1.2.3. Biochar general properties	9
1.2.4. Biochar as a technique for soil remediation and treatment	11
1.3. Dimethoate as a model-chemical case study	13
1.4. Ecotoxicological assays to assess toxicity	17
1.4.1. <i>Folsomia candida</i>	19
1.4.2. <i>Brassica rapa</i>	21
1.5. Main objectives and relevance of the study	22
1.6. Thesis organization	23
1.7. References	24
CHAPTER TWO: Scientific paper	35
2. Biochar in soil decreases the toxicity of dimethoate: a case study with the collembola <i>Folsomia candida</i> and the dicotyledonous plant <i>Brassica rapa</i>	37
2.1. Introduction	37
2.2. Material and Methods	39
2.2.1. Test soil and biochar characteristics	39
2.2.2. Test chemical and soil spiking	41
2.2.3. Test organisms	41
2.2.4. <i>Folsomia candida</i> reproduction test	42
2.2.5. <i>Brassica rapa</i> germination test	43
2.2.6. Pore water extraction	44
2.2.7. Statistical analysis	44
2.3. Results	45
2.3.1. <i>Folsomia candida</i> reproduction test	45
2.3.2. <i>Brassica rapa</i> germination test	49
2.4. Discussion	54
2.4.1. <i>Folsomia candida</i> and <i>Brassica rapa</i> as test models	54
2.4.2. Dimethoate exposure	54
2.4.3. Biochar joint application with dimethoate in soil	57
2.5. Conclusion	61
2.6. References	62
CHAPTER THREE: Final conclusions and remarks	69
3. Final conclusions and remarks	71
3.1. <i>Folsomia candida</i> and <i>Brassica rapa</i> as test models	71
3.2. Dimethoate exposure	72
3.3. Biochar joint application with dimethoate in soil	73
3.4. References	74
Supplementary data	a

List of figures

Figure 1. Representation of the pyrolysis' process. The heated decomposition (in the absence of oxygen, preferably) of the selected feedstock will result in biochar and bio-fuel. Retrieved from the website <http://www.css.cornell.edu/faculty/lehmann/research/biochar/biocharmain.html>7

Figure 2. Structural formula of the active substance Dimethoate. Retrieved from Gilbert (2014).....14

Figure 3. Adapted representation of the springtail *Folsomia candida* (PAO represents the post-antennal organ and VT represents the ventral tube). Retrieved from Fountain and Hopkin (2005).....20

Figure 4. Photograph of the dicotyledonous plant *Brassica rapa* L. from the company Carolina Biological Supply Company. Retrieved from the website http://www.carolina.com/wisconsin-fast-plants-seed-varieties/wisconsin-fast-plants-standard-brassica-rapa-seed/FAM_158804.pr#.....21

Figure 5. Glass test containers used for the *F. candida* reproduction test, performed based on the ISO procedure 11267.....42

Figure 6. Plant germination test performed using the ISO protocol 11269-2, in a plant growth room.43

Figure 7. Number of juveniles produced by *Folsomia candida* exposed for 28 days to dimethoate in LUFA 2.2 natural soil, without biochar (B0 test) and with biochar (B25 and B50 test). The grey dots indicate the number of juveniles for the test without biochar (B0 test), the black triangles indicate the number of juveniles for the B25 test and the white squares represent the number of juveniles for the B50 test. All data are presented as mean values, with standard error bars (* when $p < 0.05$ Dunnett's test, indicating statistical differences when comparing contaminated treatments with the control treatment of the B0 test). The line represents data fit to obtain the EC_{50} for the B0 test.47

Figure 8. Number of survivors from *Folsomia candida* exposed to dimethoate for 28 days in LUFA 2.2 natural soil, without biochar (B0 test) and with biochar (B25 and B50 test). The grey

dots indicate the number of adults retrieved at the end of the B0 test, the black triangles indicate the number of adults for the B25 test and the white squares for the B50 test. Data are expressed as mean values, with standard error bars (* when $p < 0.05$ Dunnett's test, indicating statistical differences when comparing contaminated treatments with the control treatment of the B0 test). The line represents data fit to obtain the LC_{50} for the B0 test.48

Figure 9. Dose-response curves for the shoot length (cm) of *Brassica rapa* after exposure to LUFA 2.2 a natural soil contaminated with dimethoate, without biochar (B0 test) and with biochar (B25 and B5 test). Grey dots indicate the shoot length for the B0 test, the black triangles indicate the shoot length for the B25 test and the white squares indicate the shoot length for the B50 test. Data are expressed as mean values, with standard error bars (* when $p < 0.05$ Dunnett's test, indicating statistical differences when comparing contaminated treatments with the control treatment of the B0 test, + when $p < 0.05$ Dunn's test, indicating statistical differences when comparing contaminated treatments with the control of the B25 test and # when $p < 0.05$ Dunn's test, indicating statistical differences when comparing contaminated treatments with the control of the B50 test). Lines represent the sigmoidal equation (logistic, 3 parameter) used to obtain the EC_{50} for reproduction.51

Figure 10. Effects on the fresh weight (mg) of *Brassica rapa* after exposure to LUFA 2.2 a natural soil contaminated with dimethoate, without biochar (B0 test) and with biochar (B25 and B5 test). Circle grey dots indicate the shoot length (mean values, with standard error bars) for the B0 test, the black triangle dots squares indicate the shoot length (mean values, with standard error bars) for the B25 test and the white square dots indicate the shoot length (mean values, with standard error bars) for the B50 test (* when $p < 0.05$ Dunnett's test, indicating statistical differences when comparing contaminated treatments with the control treatment of the B0 test, + when $p < 0.05$ Dunn's test, indicating statistical differences when comparing contaminated treatments with the control of the B25 test and # when $p < 0.05$ Dunn's test, indicating statistical differences when comparing contaminated treatments with the control of the B50 test). To fit the data and to obtain the EC_{50} for reproduction and the LC_{50} for mortality, a sigmoidal equation (logistic, 3 parameter) was selected.52

List of tables

Table 1. General and toxicological information and physical and chemical properties regarding the pesticide Dimethoate (World Health Organization n.d.; EU Pesticides database 2015; WHO Guidelines for Drinking-water Quality 2004; Pesticide Properties DataBase of University of Hertfordshire 2015).	16
Table 2. Physical and chemical characteristics of the LUFA 2.2 natural soil used in the ecotoxicological tests with collembolans and turnips.	39
Table 3. Physical and chemical properties of the selected biochar used in the reproduction test with <i>Folsomia candida</i> and in the germination test with <i>Brassica rapa</i>	40
Table 4. NOEC, LOEC, EC ₅₀ and LC ₅₀ values (mg a.i. kg ⁻¹ soil) obtained for each test, according to the biochar rate (B0: no biochar, B25: 2.5% w/w biochar incorporated with soil and B50: 5% w/w of biochar incorporated with soil). Endpoints such as reproduction and mortality of <i>Folsomia candida</i> were recorded. Test occurred for 28 days in LUFA 2.2 natural soil in the following conditions of 16h daylight and 8h dark. NOEC and LOEC were derived from a One Way ANOVA followed by Dunnet test both endpoints. EC ₅₀ and LC ₅₀ were calculated through the use of a sigmoidal equation (logistic, 3 parameter).	49
Table 5. NOEC, LOEC and EC ₅₀ (mg a.i. kg ⁻¹ soil) obtained for each test, according to the biochar rate (B0: no biochar, B25: 2.5% w/w of biochar incorporated with soil and B50: 5% w/w of biochar incorporated with soil). Parameters such as length and fresh weight of <i>Brassica rapa</i> were recorded. Test occurred for 14 days in a LUFA 2.2 natural soil. NOEC and LOEC were derived from a One Way ANOVA followed by Dunnet test for the parameter shoot length (cm) and followed by a Dunn's test for the parameter fresh weight (mg). EC ₅₀ and LC ₅₀ were calculated through the use of a sigmoidal equation (logistic, 3 parameter).	53
Table 6. Results of the Chi-square (X^2) test performed. EC ₅₀ of all tests (B0, B25 and B5 test) for both parameters (shoot length and fresh weight) were compared. (* when $X^2 > 3.84$, statistical differences between treatments exist).	53

List of abbreviations

2,4 D: 2, 4 – Dichlorophenoxyacetic acid

AChE: Acetylcholinesterase

a.i.: active ingredient

ANOVA: Analysis of Variance

BBF: British Biochar Foundation

BC: Black Carbon

CEC: Cation Exchange Capacity

EBC: European Biochar Certificate

ECBC: European Community Biochar Criteria

EC₅₀: Effective concentration for 50% of the tested population

EFSA: European Food and Safety Agency

EPA: Environmental Protection Agency

EqPT: Equilibrium Partitioning Theory

ERA: Ecological Risk Assessment

EU: European Union

FAO: Food and Agriculture Organization of the United Nations

IBI: International Biochar Initiative

ISO: International Organization for Standardization

LC₅₀: Lethal concentration for 50% of the tested population

LOEC: lowest observed effect concentration

MoA: Mode of Action

NOEC: No observed effect concentration

OECD: Organisation for Economic Co-operation and Development

OP: Organophosphorous

PAH: Polycyclic Aromatic Compound

PAO: Post-Antennal Organ

PBT: Persistence, Bioaccumulation and Toxicity

PCB: Polychlorinated Biphenyl

PTE: Potentially Toxic Element

RCF: Root Concentration Factor

VT: Ventral Tube

WHC: Water Holding Capacity

CHAPTER I

GENERAL INTRODUCTION

1. General Introduction

1.1. Soil contamination

Soil is the most external layer of Earth's crust and can be defined as a natural body formed at the surface of Earth with layers composed by water, air, organic and mineral materials, where organisms interact (FAO Soils Portal n.d.; Soil Science Society of America n.d.). It is responsible for numerous vital functions like biomass production (including food), filtration, transformation of some substances and nutrients, habitat provision, or soil structure maintenance (Soil Science Society of America n.d.), and it is a non-renewable resource, whose constitution is very heterogeneous (Commission of the European Communities 2006; van Gestel 2012). Soil can be considered as a key piece for terrestrial ecosystems, because of the importance it has for nutrients, microorganisms, flora and fauna and for the humankind.

The Industrial Revolution was responsible for the development of some industrial technologies that caused the release of several types of pollutants to the environment, leading to soil, air and water contamination (Fagervold et al. 2010; Fornes et al. 2009; Beesley et al. 2010; Ahmad et al. 2014). Considering that soil contamination has been a widespread problem and due to the lack of European Union (EU) legislation about soil protection (European Commission 2013), there is a need to find and develop technologies that can minimize or solve it. According to the EU Commission (2013), there are approximately three million places possibly affected by soil contamination and 250,000 of those places need remediation as soon as possible. So, due to the importance soils represent for the ecosystems, the 68th United Nations General Assembly on 20th December of 2013 stated 2015 as the International Year of Soils. With this initiative, they pretended to inform and educate the population about the importance soil has for human life and to promote soil management through the development of new policies and actions (Food and Agriculture Organization of the United States n.d.).

Soil contamination has increased mainly because of the rise of human population, whose activities increase the release of xenobiotics to the environment, but who demands

also more food and, therefore, more soil productivity. Agricultural practices, which include the application of pesticides, can be one of the main causes of soil contamination (Amorim et al. 2012). Groundwater contamination, health problems, deterioration and loss of soil functions (European Commission 2013) are the major complications associated with this problem. The levels of contamination can vary with the depth of soil and can affect the soil quality and fertility (European Commission 2013). Some physical and chemical processes (e.g. redox reactions and precipitation) can affect the behaviour of a chemical in the soil and, besides these, the contaminant can also be sorbed or become available in soil suspensions, depending on soil texture and structure (type of mineral present, the amount of organic material, the soil's pH, the redox potential and the type of moisture) (European Commission 2013). A healthy soil has specific physical, chemical and biological properties and if it is contaminated, its performance will be affected and, thus, some key functions can be equally jeopardized. Therefore, it is urgent to keep in mind that soil properties can vary from place to place; in other words, each local has an unique risk profile, an unique chemistry and an unique history (European Commission 2013). For these reasons, the bioavailability of a chemical can vary with the soil type, environmental conditions but also with soil biota, which complicates the gathering of data to create guidelines and legislation about soil contamination.

As previously mentioned, the introduction of chemical substances leads to effects on human health, on agricultural productivity and on the ecosystems. Soil contamination is a crucial issue in environmental protection goals although it has majorly been considered as an insoluble problem. Therefore, creating solutions for soil remediation are urgent and need to be tackled carefully. Soil remediation is important because soil unavailability represents a problem with direct consequences (Sousa et al. 2008) and it provides ecosystem services that enable life on earth, like food production, water quality and supply, climate regulation, pollution attenuation and degradation, pest and disease control or biodiversity conservation, which it is vital for the humankind (Barrios 2007; Adhikari & Hartemink 2016). Pesticides were and are being exhaustively used to improve crop production and it is important to keep in mind that human wellbeing is connected with soil; so, if it is contaminated and unsafe, we will be largely affected. It is almost impossible to remediate a polluted soil to its safe and original state but it can be technologically viable; however, it can be overpriced and can damage some characteristics of soils (Fornes et al. 2009; Lemming et al. 2010; Suèr et al. 2004).

As stated before, even though soils contribute to ecosystem's services, there is no legislation aiming their protection (European Commission 2013). Even though, remediation methods are used to protect soils, to improve their quality and to avoid more environmental problems and risks for human health (European Commission 2012).

These treatment methods can be grouped into physical, chemical or biological remediation, according to the best approach for the specific case to be treated. Physical treatments include the removal of hazard(s) through physical means, as thermal desorption, soil replacement, soil washing and capping, while chemical treatments use chemical agents and chemical reactions to remove the hazard(s), like chelators, chemical immobilization and oxidation. Biological treatments, such as phytoremediation (plants) or bioremediation (microorganisms), usually involve the use of living organisms to reduce chemical concentration and/or hazard(s)' risk. Besides, these treatments can also be applied *in situ* or *ex situ*, which is the treatment in the place without its removal and the removal of the contaminated place to a treatment site, respectively (EPA 2006). Biochar is an environmentally acceptable remediation' method that, besides its function as soil amendment, can act *in situ* to decrease the bioavailability of some pollutants to the soil biota.

1.2. Biochar

1.2.1. Introducing biochar: a brief historical record

Nowadays, there are three main challenges that the humankind needs to solve: the climate change, the food crisis and the energy crisis. Some scientists believe that biochar is the answer, as it combines within its function carbon retainer, soil fertility improver and as a soil amendment (Mylavarapu et al. 2013; Ahmad et al. 2014; Cabrera et al. 2014); for those reasons, biochar application is increasing exponentially (Oleszczuk et al. 2013; Lehmann & Joseph 2015).

According to Verheijen et al. (2010), biochar is, basically, charred organic matter produced for soil application. In a more complete and scientific definition, biochar can be classified as the product of biomass pyrolysis, a process that involves the thermal degradation (from 300 °C to 1000 °C) of a certain type of biomass in an oxygen-limited

environment (preferably null), resulting in (besides the solid product (biochar)) non-condensable vapours and combustible bio-oil (Lehmann & Joseph 2015; IBI n.d.). It is enriched with carbon, phosphorous and sometimes calcium, magnesium and nitrogen, depending on the type of feedstock (Verheijen et al. 2010). The big difference between biochar and charcoal relies on the fact that biochar is produced only for soil application and charcoal is used to produce energy and fuel (Ahmad et al. 2014; Wiedner & Glaser 2015), and not with the intention to be added to soil (Lehmann & Joseph 2015). Biochar can be characterized as sustainable and viable, with benefits for the atmosphere and for the soil, like the improvement of agriculture crops, carbon sequestration, waste management and production of clean energy (Lehmann & Joseph 2015).

Biochar has been used as a pyrogenic carbonaceous material in many parts of the world since at least 5000 years ago (Wiedner & Glaser 2015; Spokas et al. 2012). These carbonized materials have been playing an important role since ancient intensive agriculture (Wiedner & Glaser 2015), so biochar application in soils is not a new concept (Lehmann et al. 2006).

The Amazonian soil is characterized as barely fertile and highly weathered but a small area within Amazonia known as Amazonian Dark Earths (or “*Terra Preta do Índio*”) is highly fertile, with different properties compared to the other types of soils in the region (Glaser & Birk 2012). Consequently, this small area can take the responsibility for the worldwide interest and current application of biochar to soil (Wiedner & Glaser 2015; Spokas et al. 2012; Ahmad et al. 2014), as it revealed the power and the effects biochar had on Amazonian soils (Ahmad et al. 2014). This man-made land (Verheijen et al. 2010) was created by the aboriginal inhabitants where a massive input of organic waste materials and charred residues was decomposed by soil organisms (Wiedner & Glaser 2015; Brick 2010). However, the aim of its application remains unclear, whether it was on purpose or accidental (Lehmann et al. 2006; Brick 2010; Wiedner & Glaser 2015). These findings showed that charcoal seemed to improve the soil’s structure by changing both the soil’s chemistry and ecology. Besides, it also suggested that those lands had an important role in mitigating the climate change since it contained high concentrations of carbon that have been stabilized for years (Brick 2010). So, what happens in “*Terra Preta do Índio*” provides information about long term consequences (Verheijen et al. 2010) that can help society improve the conditions and type of feedstock used in the future. Some improvements are being developed in order to get biochar with

greater quality from the pyrolysis for application procedures and different aims (Lehmann & Joseph 2015).

1.2.2. Biochar production

The biochar technology industry can possibly be improved simply by combining the specific biochar with the problems to solve or by developing biochars for the exact application (Kleber et al. 2015). This is possible, because, by controlling the pyrolysis temperature and style, the desired product can be designed and obtained (Ippolito et al. 2015).

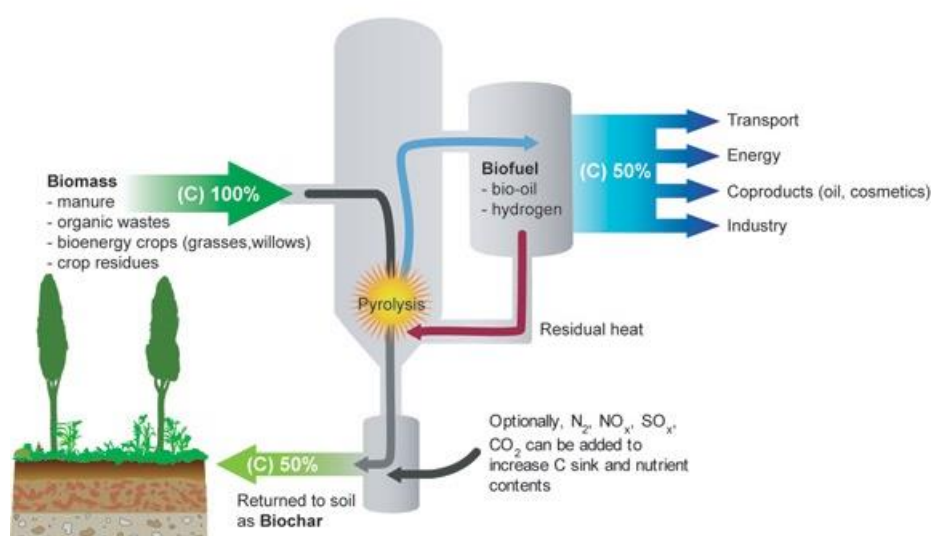


Figure 1. Representation of the pyrolysis' process. The heated decomposition (in the absence of oxygen, preferably) of the selected feedstock will result in biochar and bio-fuel. Retrieved from the website <http://www.css.cornell.edu/faculty/lehmann/research/biochar/biocharmain.html>.

Biochar production begins with pyrolysis (Fig. 1), a recognized and long standing technology (Lehmann & Joseph 2015) capable of producing non-condensable gases, combustible bio-oil and biochar as the solid residual coproduct for carbon sequestration (Boateng et al. 2015; Spokas et al. 2012). According to Lehmann (2007), by combining the biochar sequestration with bioenergy production, a clean energy technology will be attained with the reduction of greenhouse gas emissions and carbon sequestration. Nowadays, modern conversion systems can control the operating systems which allow, along with the finest selection of the feedstock, the regulation of chemical and physical

properties of biochar, resulting in the customization of biochar properties (Spokas et al. 2012). Pyrolysis technologies have been trying to change the pollutant problem of the feedstocks, like the increase of potentially toxic elements' (PTE) concentrations and the formation of polycyclic aromatic compounds' (PAH) or dioxins (Domene et al. 2015a). The type of pyrolysis, along with the long list of feedstocks available, can result in different samples of biochars in terms of composition; this includes biochars that can be appropriate (or not) for soil amendment and not useful for other objectives, like carbon sequestration for example (Domene et al. 2015a; Ahmad et al. 2014).

Generally, black carbon or carbon black (BC) – a pyrogenic carbonaceous material dispersed in the environment from wildfires and fossil fuel combustion (Lehmann & Joseph 2015) - has been an undesirable waste product as the industry's primary focus is to optimize the liquid and gas products and not the production of biochar for carbon sequestration (Spokas et al. 2012). In order to obtain the most environmental and economic benefits from biochar production, an enhanced charring and pyrolysis technologies, which can offer compromise between high yield and biochar with good quality, along with the capability of producing heat from the combustion of pyrolysis vapours or recovering gaseous and liquid co-products, is required (Boateng et al. 2015). Fortunately, there have been some improvements in energy efficiency and a reduction in pollution emissions, being possible nowadays to modify biochar properties to accomplish the agronomic and carbon sequestration applications (Boateng et al. 2015). A better use of pyrolysis co-products might improve economic prospects of biochar production (Boateng et al. 2015).

There are several types of biomass, such as agriculture by-products, industrial by-products, animal wastes and sewage sludge, which can produce a wide variety of biochar materials, each with its own opportunities and constraints (Lehmann & Joseph 2015; Mylavarapu et al. 2013; Brick 2010). In other words, the quality of the feedstock can influence the final biochar product (Ippolito et al. 2015; Jeffery et al. 2015; Verheijen et al. 2010). Using products from waste biomass sources (such as animal waste, municipal waste and sewage sludge) can come along with some problems, since they can have some hazardous components due to the source of these feedstocks (Verheijen et al. 2010).

Basically, the nature of feedstock and the type of pyrolysis conditions are what makes the different types of biochar exist – each one with different characteristics -, being

these characteristics important for the selection of the type of application (Domene et al. 2015a). Pyrolysis occurs at high temperatures - between 300 °C and 1000 °C -, depending on the moisture content of the biomass (Verheijen et al. 2010) and, when the biomass is heated to a point where this process occurs, the energy generated is sufficient to continue the reaction (Lehmann & Joseph 2015). The heat rate is also important to determine the pyrolysis type of production: when low (known as slow pyrolysis), the biochar production is maximized, and when high (known as high pyrolysis), the bio-oil production is maximized (Boateng et al. 2015).

1.2.3. Biochar general properties

As mentioned before, biochar's physical properties are influenced by its structure, which depends on the feedstock and pyrolysis conditions (especially the process temperature and the heating rate). Consequently, when biochar is added to soils, some interactions can occur and, therefore, cause effects on some soil properties, which can alter some ecosystems components (Chia et al. 2015).

Structurally, biochar is very heterogeneous (Verheijen et al. 2010). Almost every type of biochar has a high content of carbon and a high aromatic ring structure, due to the high thermal degradation (Kleber et al. 2015). However, they can differ because of the different types of feedstock used and different pyrolysis conditions chosen. Chemically, biochar is constituted by carbon, volatile matter, ash and moisture (Verheijen et al. 2010). In fact, the chemical structure of the surface area is important because it can explain why biochar can interact with other composts (Chia et al. 2015), such as pesticides. Also, the particle size and the pore size distribution can both be influenced by the type of feedstock, as well as by the conditions of pyrolysis performed, and both will influence the application of biochar in soils (Chia et al. 2015). Both cation exchange capacity (CEC) and pH can be influenced by the pyrolysis temperature and type and these factors can influence the application of biochar in soils, as high CEC and high pH (biochar normally has a neutral to basic pH) can improve the retention of nutrients and the productivity of acidic soils (Ippolito et al. 2015).

It is safe to say that the proportion and the arrangement of the physical, chemical and structural characteristics of biochar components determine its behaviour in soils and,

therefore, influence its application and the agronomic results (Spokas et al. 2012). It is important therefore to study the different structures of the several types of biochar before soil application. Some of the biochar properties are related directly to its persistence in soil, with longer residence time. However, there is no knowledge on the precise duration of biochars' storage time (Lehmann 2007) and there are some speculations that this trait is due to its low mineralization, which depends on abiotic factors such as moisture, temperature and soil properties, and on biotic factors such as the decomposition by microorganisms (Lehmann et al. 2015).

To sum up, when biochar is incorporated in soils, these can suffer some changes in the structure, density and pore size distribution, which can later lead to implications in soil aeration, water holding capacity (WHC) and plant growth (Verheijen et al. 2010). Different types of biochar will lead to different reactions in crops productivity.

As stated before, biochar can also represent a source of contaminant residues due to feedstocks used and to the conditions of pyrolysis, and, for this reason, it can have effects on soil biota and even on human health. Metallic elements and organic pollutants like PAHs, polychlorinated biphenyls (PCBs), furans, dioxins and others may appear in biochar, but always within admissible values regarding some standards (established to protect the environment) (Hale et al. 2012; Ahmad et al. 2014; Buss & Mašek 2014). Some organizations such as International Biochar Initiative (IBI), European Biochar Certificate (EBC) and the British Biochar Foundation (BBF) are collaborating for the development of standards to guarantee a sustainable biochar production, utilization and quality, for any nation (European Biochar Certificate 2012; International Biochar Initiative 2015; British Biochar Foundation 2014; Domene et al. 2015a).

Since biochar has caught the attention of several countries, due to its advantageous potentialities, the concern about the risks these pollutants have for soils has been rising (Verheijen et al. 2010). It is essential to understand what will be the bioavailable fraction for different organisms and what effects it may cause. As a matter of fact, the occurrence of these chemicals constitute a problem to public health, since these are toxic for humans and we can be exposed during the manufacturing, the application and even through contaminants that can be incorporated in food. However, there are not any toxicological reports about the PAHs incorporated in biochar (Verheijen et al. 2010) and we can only speculate about their effects in human health.

It is important to take into account the importance of soil biota for the ecosystem and, more important, is the study of effects induced by different types of biochar to the biota (Lehmann et al. 2011). So, before there is a decision on whether biochars can be used as soil amendment, their hazard assessment should be carried out to define limits for application. In fact, the information about the interaction between soil biota and soil amended with biochar is still scarce. The type of biochar, the pyrolysis procedure and the rate of biochar addition can be the factors that explain the biota's responses to biochar, along with the type of soil and the possible changes in soil properties (Domene et al. 2015b; Marks et al. 2014; Oleszczuk et al. 2014).

1.2.4. Biochar as a technique for soil remediation and treatment

Besides the several advantages that biochar can bring to improve soil quality, mainly by changing soil structure, it can also be used for other purposes, like remediation processes. When it comes to soil remediation, some available techniques are not fully considered due to their high costs, but also because they can generate impacts due to some actions of these remediation activities (Janus et al. 2015; Lemming et al. 2010). In the past few years, new low impact and cost-effective remediation techniques have been developed (Brennan et al. 2014), such as the application of low cost organic amendments (Beesley et al. 2015), in order to reduce the pollutant bioavailability and toxicity (Hale et al. 2015), maintaining a sustained functioning of the ecosystem functions (Thies et al. 2015).

Aside its function as soil amendment, biochar can be used as well for sorption of pollutant compounds and, thus, treatment and restructuration of contaminated soils (Pignatello et al. 2015).

Thies et al. (2015) suggest that biochar is capable of adsorbing toxic compounds, like pesticides, preventing changes in the abundance, diversity and activity of soil organisms. According to Verheijen et al. (2010) and Hale et al. (2015), biochar, by remediating a pollutant, will influence the toxicity of a chemical by affecting its accessibility, availability, transport and fate; however, this sorption will depend on the biochar production and on its physical and chemical properties (Janus et al. 2015; Oleszczuk et al. 2014). The remediation begins with desorption of the pollutant from the

contaminated spot, followed by the migration of the molecule to the biochar particle and, in the end, this particle will sorb the molecule into its network (Hale et al. 2015). So, hopefully, the active ingredient will be reduced in its bioavailable form and it will be less toxic to the soil biota and flora. However, scientists suggest more investigation regarding the interaction of physical and chemical properties of biochar with the sorption ability (Verheijen et al. 2010; Oleszczuk et al. 2014), because for an effective remediation, the biochar power as a sorbent has to be stronger than the soil matrix (Hale et al. 2015), where time and environmental/climate conditions will also play a key role.

The big question that arises from the use of biochar to remediate contaminated soils and that scientists try to solve is if this technique is sustainable, because, since biochar will accumulate the pollutant, we will not know what its behaviour will be. In other words, there is no knowledge about whether it will result in an infinite accumulation with no problems for the ecosystem or if, someday, it will disintegrate and cause detrimental problems for the ecosystem and for the public health. The answer to this question, according to Thies et al. (2015), could depend on the number of biochar binding places on the surface that allows the sorption of the molecule, on the presence of other molecules that can compete for these places, on the biochar properties and on the access that soil fauna can have to the chemical. In addition, another problem enters in the equation: the biochar “ageing”. Considering there are some questions about the behaviour of biochar over a long period of time and how the interactions between biochar and soil will be influenced, there is not much certainty about what will happen with pollutants sorbed in time, if they will be available again (and more toxic) or not. However, it is undeniable that the use of biochar as a remediation technique can mitigate the toxicity and transport of pollutants and, therefore, improve soil quality (Verheijen et al. 2010). Considering these concerns, biochar application can be of major importance for organic chemicals, like pesticides, that are design to act immediately upon application, presenting also low half-life rates. By immobilizing the substance right after application, this will potentially prevent further hazard of the parental compound.

1.3. Dimethoate as a model-chemical case study

According to the European Food and Safety Agency (EFSA), a pesticide can be described as a product that protects plants, with the main goal to keep crops healthy and protect them from diseases and infestations (European Commission 2015b). These plant protection products, which are being used to control pests and disease vectors, are also toxic to non-target organisms and are being extensively used in agriculture, so it is urgent to assess the risk they could represent for ecosystems (Basant et al. 2015; Graber & Kookana 2015; Martikainen 1996).

Dimethoate (Fig. 2) was considered in the Edition of 2007 of Eurostat Statistics Book as the third active substance most used in the European Union (Eurostat 2007). It is one of the most widely used insecticides sold in the entire world (Pesticide Action Network UK 2002) and it was approved in October 1st of 2007 for consume in the majority of the member states of the European Union (except in Denmark, Lithuania and Latvia) (EU Pesticides database 2015). In Portugal, according with the Phytopharmaceutical Products' Guide developed by *Direção Geral da Alimentação e Veterinária do Ministério da Agricultura e do Mar*, in which all products with authorized sale are listed, dimethoate is approved for sale in the form of concentrate for emulsion although it is classified as harmful and dangerous for the environment (Direção-Geral de Alimentação e Veterinária 2015). Due to its efficiency and fast environmental degradation, it is used worldwide both in agriculture, against a broad range of insects (e.g. the control of the housefly) and in urban areas (International Programme on Chemical Safety 1989). It was originally produced and patented in the 1950s by the American Cyanamid Company (Farm Chemicals International 2010), being nowadays commercialized by various brands; so it is a xenobiotic as it does not appear naturally in the environment (International Programme on Chemical Safety 1989).

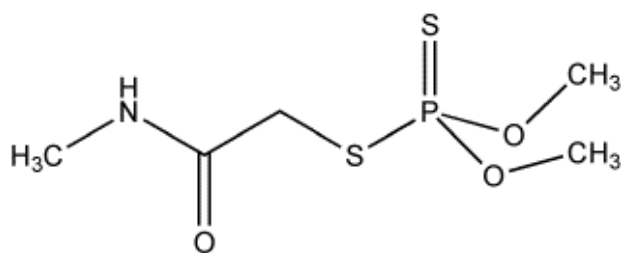


Figure 2. Structural formula of the active substance Dimethoate. Retrieved from Gilbert (2014).

Dimethoate is an insecticide that belongs to the class of organophosphorus (OP) compounds, which has the ability to inhibit acetylcholinesterase (AChE), an important enzyme for the functioning of the nervous system of mammals, fish, birds and insects (Van Scoy et al. n.d.), capable of degrading the acetylcholine (a neurotransmitter) in acetic acid and choline (Walker et al. 2001). The OP insecticides, because of its mode of action (MoA), cause a continuous stimulation and an excessive accumulation of acetylcholine in cholinergic synapses, triggering cholinergic toxicity and, consequently, death (Casarett & Doull 2008). This pesticide is constituted by organic esters of phosphorous acids and, generally, are polar and water soluble; therefore, the significant solubility and the hydrophilic capacity are essentials to allow the entrance in the nervous system (Walker et al. 2001).

The insecticide is used to kill insects, such as mites, flies, plant hoppers and aphids, by contact and systemic action (Gilbert 2014; Pesticide Action Network UK 2002; European Food Safety Authority 2013). It can be applied in various types of crops, such as vegetables and fruits, and in landscape maintenance and pest control (Van Scoy et al. n.d.).

The physical and chemical properties are resumed and described in table 1. This pesticide consists of a white solid powder with a characteristic odour to mercapturic, having a mix of dimethoate (active substance) with, sometimes, some impurities (due to manufacturing) (World Health Organization n.d.).

There are some properties expressed in table 1 considered very important to classify dimethoate, in terms of environmental studies. For example, the solubility in water at 20 °C when above 0.5 g L⁻¹ means that the chemical is easily dissolved in water; by observing table 1, we perceive that dimethoate can be well dissolved. The coefficient octanol-water (expressed in log) shows the ability of a given chemical to cause bioaccumulation and,

when below 2.7, it is stated that the chemical does not cause bioaccumulation. Due to dimethoate hydrophilicity (table 1), dimethoate is not prone to be bioaccumulated by the biota. The Henry's law constant show the volatility of a chemical and if the value is below $0.1 \text{ Pa m}^3 \text{ mol}^{-1}$, it means that the chemical in question is non-volatile. So, in this case, we can state that dimethoate is non-volatile (Pesticide Properties DataBase of University of Hertfordshire 2015).

Table 1. General and toxicological information and physical and chemical properties regarding the pesticide Dimethoate (World Health Organization n.d.; EU Pesticides database 2015; WHO Guidelines for Drinking-water Quality 2004; Pesticide Properties DataBase of University of Hertfordshire 2015).

ISO common name	<i>O,O</i> -dimethyl <i>S</i> -methylcarbomoylmethyl phosphorodithioate
IUPAC nomenclature	2-dimethoxyphosphinothioylthio- <i>N</i> -methylacetamide
CAS number	60-51-1
Molecular weight (g mol ⁻¹)	229.26
Chemical formula	C ₅ H ₁₂ NO ₃ PS ₂
Physical state	White coloured crystals
Density (g mL ⁻¹)	1.31
Solubility in water at 20 °C (g L ⁻¹)	39.8
Degradation point (°C)	113
Octanol-water partition coefficient at pH 7, 20°C (log K _{ow})	0.704
Henry's law constant at 25°C (Pa m ³ mol ⁻¹)	1.42x10 ⁻⁰⁶
Half-lives in aqueous solutions (days)	
pH 2-7	Stable
pH 9	12
Major degradation product	Omethoate

When classifying a chemical, it is important to follow the PBT (persistence, bioaccumulation and toxicity) criteria. These three factors usually diagnose the level of toxicity of a chemical. We can see if a chemical is persistent or not by checking its half-life and, for dimethoate, it is safe to say that this chemical is not persistent as it is easily degraded in basic pH and stable when in a solution with an acid pH (Ferreira et al. 2015). In the environment, dimethoate can be easily degraded by hydrolysis (main inactivating pathway) depending on pH, soil type, temperature and other abiotic factors, and by photolysis (Van Scoy et al. n.d.). Dimethoate can also be degraded by biotic factors, such as microbial degradation. Actually, this degradation, played mainly by bacteria, is responsible for reducing most part of pesticides residue existent in the environment (Van Scoy et al. n.d.). The second factor, bioaccumulation, was already addressed in the last paragraph. By evaluating the intensity of the adverse effects this chemical can cause in organisms, we can discuss the last topic, toxicity. With dimethoate, aquatic organisms and birds can be moderately to highly affected, and honeybees can be seriously affected (International Programme on Chemical Safety 1989). For soil organisms there are also several studies highlighting the potential effects of dimethoate at levels similar to the application rates (e.g. Ferreira et al. 2015; Santos et al. 2010).

Therefore, in the present study, this pesticide was chosen as chemical model as will be explained further in the objectives section of this chapter.

1.4. Ecotoxicological assays to assess toxicity

Chemicals are present in large scale in our lives and, most of the times, can damage severely the human health and/or the environment (European Commission 2015a). The Environmental Protection Agency (EPA) defines risk as “*the chance of harmful effects to human health or to ecological systems resulting from exposure to an environmental stressor*”. This stressor can be of physical, chemical or biological nature (European Commission 2015a). To evaluate the possibility of the occurrence of adverse ecological and health effects in organisms as a result of exposure to hazardous stressors and ensure a high level of protection (European Commission 2015a), Environmental Risk Assessment (ERA) is implemented (Casarett & Doull 2008). In this scientific process, some critical elements obtained from toxicological research and toxicity testing were

provided in order to characterize the magnitude of risks to humans and other organisms (USEPA n.d.; Casarett & Doull 2008).

The awareness about the risks some human activities cause to the environment and to human health required scientists to discover how to predict the consequences of releasing chemicals to the environment (Altenburger & Schmitt-jansen 2003). So, in order to assess and study the toxicity of chemicals and the intensity of adverse effects caused by them, ecotoxicological assays were developed and nowadays are used to test the influence of substances on organisms. Soil and water contamination can be detected by analytical chemistry; however, these analytical methods do not distinguish the bioavailable fractions from those that are not available to organisms, and therefore induce no effects. Recent advances showed the interest in the assessment of the contaminant availability as well in the development of ways to reduce the bioavailable fraction (Brennan et al. 2014). Bioassays are important tools to study the bioavailable fractions of substances (Domene et al. 2015a), either in soils or water, because they focus on the bioavailable fraction. The Equilibrium Partitioning Theory (EqPT) is a model based on the hypothesis that the bioavailable dissolved fraction of a chemical in the interstitial water is what may cause toxicity to organisms in sediments and soils (OECD 1992; van Der Kooij et al. 1991; Di Toro et al. 1991; Belfroid et al. 1995; Lima et al. 2011). The octanol-water partition coefficient ($\log K_{ow}$) can give information about the solubility of a chemical in the water. Therefore, by knowing this chemical property, it is possible to determine if the chemical is adsorbed to the sediment particles or if it is bioavailable on the interstitial water for uptake by the organisms (Belfroid et al. 1995).

The Organization for Economic Co-operation and Development (OECD) and International Organization for Standardization (ISO) are non-governmental organizations responsible for developing methods internationally accepted that can assess the possible effects of chemicals on the environment and/or in the human health. Focusing on the effects on biotic systems, there are several toxicity tests with numerous test organisms. When developing the experimental design of an assay, the type of test we want to use should be remembered, as well as the organisms. It is very important to incorporate ecological relevance in the toxicity tests (Bogomolov et al. 1996) and, the higher the organization level, the more ecologically relevant are the results. These standardized bioassays as well from other non-standardized bioassays can provide as final output several parameters derived from dose-response curves like EC_{50} (effective concentration

that affects 50% of the population tested), LC₅₀ (lethal concentration that affects 50% of the population tested), NOEC (the highest concentration tested that does not cause any effect to the population tested) and LOEC (lowest concentration tested that cause an effect to the population tested) should be expressed. These parameters are then used to describe and assess hazard and compiled to be used in risk assessment procedures.

For a soil to be considered healthy it is essential to protect fauna, as it is responsible for the decomposition of organic matter, regulation of microbial activities, nutrient cycles and even for the structure (Cortet et al. 1999). When a disturbance in the soil occurs, the fauna can suffer quantitative and/or qualitative changes, and these can be responsible for stressing a soil health. These biological responses can be used to connect the contaminant toxicity with ecotoxicological responses.

Knowing that chemicals can affect individual organisms (Maltby 1999), several terrestrial organisms and aquatic organisms are used in toxicity tests to evaluate the hazard and risk of a chemical in ecosystems. Focusing on terrestrial organisms, soil invertebrates – like collembola, earthworms and isopods, among many others - and terrestrial plants - monocotyledons and dicotyledons plants - belong to the most commonly test organisms with standardized protocols used in soil ecotoxicology, especially because they play important roles in several ecosystem services. The most common endpoints evaluated in soil invertebrates are avoidance, survival and reproduction, being this last parameter the most relevant, because it can provide an idea about potential effects to a population level (van Gestel 2012). When it comes to plants, the most common endpoints tested are seedling emergence, fresh weight and dry weight, shoot height and visualization of detrimental effects on the plant (OECD 2003).

1.4.1. *Folsomia candida*

Springtails are among the most used arthropods for ecotoxicity testing (Cortet et al. 1999) because they are considered representative of the soil mesofauna (Domene et al. 2015b). Fountain and Hopkin (2005) referred collembolans are one of the most abundant arthropods and an essential part of soil ecosystems. They are distributed worldwide, but prefer habitats very rich in hummus and organic matter, since they are decomposers (OECD 2009). Fountain and Hopkin (2005) consider *F. candida* as a tramp species, since

it has been spread around the world inadvertently by human commerce and there is a lack of information regarding the original biogeographic location, though some records refer their presence in places like caves and mines. They can, also, be very affected by episodes of contamination and, in this way, be used also as bioindicators.

Folsomia candida Willem 1902 (Fig. 3) is a white eyeless springtail that can measure up to 3.0 mm, when adult (Fountain & Hopkin 2005). They have the body divided in three sections – the head, the thorax and the abdomen, covered with a thin exoskeleton permeable to air and water (OECD 2009). The post-antennal organ (PAO) (Fig. 3), located in the head behind the base of each antenna, can, supposedly, detect chemicals in the air. The ventral tube (VT) (Fig. 3) in the first abdominal section is responsible for being an exposure route to chemicals dissolved in the pore water of soils. Springtails do not possess respiratory pigments neither trachea, so their oxygen capacity is very low and their uptake is by the cuticle (Fountain & Hopkin 2005) .

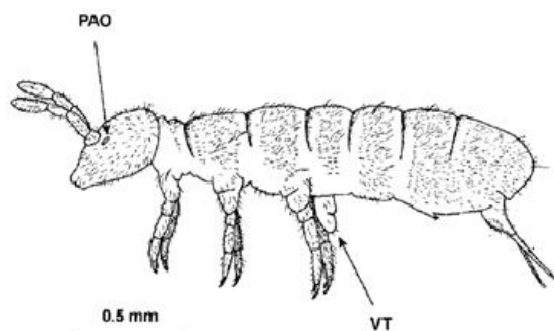


Figure 3. Adapted representation of the springtail *Folsomia candida* (PAO represents the post-antennal organ and VT represents the ventral tube). Retrieved from Fountain and Hopkin (2005).

F. candida is a parthenogenetic organism, with most of the population members being females (males are extremely rare) (Fountain & Hopkin 2005; Krogh 2008).

Springtails can be exposed to contaminants by several ways: via soil, food gas, pore water (principal and most toxic route of exposure), contaminated leaf surfaces and topical application onto the individual. As they are a part of soil fauna, they are used as standardized organisms' models to assess soil toxicity of different pollutants. Along with earthworms, springtails are one of the most used organisms in ecotoxicological assays because of its representativeness and widespread distribution, very easy to maintain in

laboratory cultures with controlled conditions and to manipulate along the experimental procedure, as well as with standardized ecotoxicological guidelines.

1.4.2. *Brassica rapa*

Besides soil invertebrate tests developed by OECD and ISO, these organizations have also developed toxicity tests with plants, which are important in soil ecosystems as primary producers. The application of pesticides can affect non-target plants, so, any influence can have serious impacts and consequences in the ecosystem, affecting other organisms.



Figure 4. Photograph of the dicotyledonous plant *Brassica rapa* L. from the company Carolina Biological Supply Company. Retrieved from the website http://www.carolina.com/wisconsin-fast-plants-seed-varieties/wisconsin-fast-plants-standard-brassica-rapa-seed/FAM_158804.pr#.

The family *Brassicaceae* constitutes one of the most economically important plant groups worldwide and includes the common turnip specie *Brassica rapa* L. (Fig. 4), which has been used as model by botanists in several areas of research (Kelly 2006; Williams & Hill 1986). These plants were originated in the mountainous areas around the Mediterranean sea (Eurasia) and they are able to survive and grow in places with low

temperatures, which allows the cultivation in cool temperature regions; nowadays, they exist all over the world, and their life cycle is annual (OECD 2012) .

B. rapa has a short life cycle with seed germinating after three or four days and a rapid fall growth, a high biomass production and can provide shelter for insects in the environment (OECD 2012). It prefers well-drained, moist soil but it can grow in other types of soil, such as soils with low fertility and in droughty conditions (Young-Mathews 2012). Regarding reproduction, the flowers are regular, bisexual and hypogynous and its pollen is airborne and can float in the air or being transferred by pollinators (Young-Mathews 2012). In terms of toxicity, *B. rapa* is used frequently in standardized ecotoxicological bioassays and the endpoints that are normally measured are the plant's germination, fresh and dry weight, as well the shoot length (ISO 1995). These responses are closely related to the physiological status of plants, and changes in normal patterns occurring in these plants will lead to changes in soil functions and ecosystem services.

1.5. Main objectives and relevance of the study

Considering all the above mentioned information, the proposed hypothesis in this thesis is that biochar application to a contaminated soil can be used as a remediation tool in agroecosystems. Therefore, the main goal of the present study was to evaluate the toxicity of dimethoate (chosen as a model-agrochemical) to the collembolan *Folsomia candida* and the plant *Brassica rapa*, under three scenarios: alone and with two biochar amendments (in terms of volume application).

As previously mentioned, there is a vast area of contaminated soils in need of treatment. In addition, decreasing deleterious effects upon agrochemical applications is also desirable. Even though biochar is used as a soil amendment, it can also be used as a remediation technique. However, there is not much information concerning this function. The use of non-target invertebrates and non-target plants consists can provide an accurate indication of the efficacy biochar application may have in a contaminated soil. By using standardized protocols, results can be easily compared to others already available in the literature and therefore strengthen the results achieved. The two organisms chosen are known to be exposed to contaminants mainly through soil pore water. Therefore, the

hypothesis stated above could be confirmed by evaluating the dimethoate concentration in pore water and looking at results based on the Equilibrium Partitioning theory.

1.6. Thesis organization

The present dissertation is divided in three chapters, with the second chapter structured as a scientific paper.

- **Chapter I:** General Introduction, Research Aims and Relevance.
- **Chapter II:** “Biochar amendment in dimethoate contaminated soils: toxicity assessment using the collembolan *Folsomia candida* and the dicotyledonous plant *Brassica rapa*”
- **Chapter III:** General Discussion, Concluding Remarks and Recommendations.

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CHAPTER II

Scientific paper

2. Biochar in soil decreases the toxicity of dimethoate: a case study with the collembola *Folsomia candida* and the dicotyledonous plant *Brassica rapa*

2.1. Introduction

Anthropogenic activities like agricultural practices, urban activities and industrial processes along with natural disasters are the main causes responsible for soil contamination (Lima et al. 2011; Yang et al. 2006). They can result in groundwater contamination and health problems (European Commission 2013), along with all the deterioration and loss of soil functions. Although the pesticide industry is a component of the world agriculture (Sun et al. 2012), there are some struggles between agriculture and environmental quality and protection because agrochemicals – responsible for the maintenance of crops, by controlling pests and diseases (Tang et al. 2013) -, can sometimes cause episodes of contamination (Loureiro et al. 2009). Even though pesticides are an important component of intensive world agriculture (Sun et al. 2012; Jones et al. 2011) soil can act as a sink for those toxic compounds.

Some remediation techniques are considered technologically viable (Fornes et al. 2009) but, at the same time, inappropriate since they can disturb the environment and be expensive, especially in a large scale (Janus et al. 2015). Since soil is a fundamental resource for agriculture (Safaei Khorram et al. 2015), cost-effective and environmental alternatives to the unsustainable and common waste disposal techniques are being developed to treat soil contamination (Beesley et al. 2011; Safaei Khorram et al. 2015; Sopena et al. 2012).

BC is a new engineered sorbent for environmental application (Chen & Chen 2009) made of carbonaceous materials like chars, charcoals and biochars, with positive effects on the sorption, degradation and bioavailability of pesticides to fauna and flora (Nag et al. 2011). For years, researchers have been investigating technologies that can produce bioenergy, remove excessive carbon from the atmosphere and improve both water, soil and air quality (Kleber et al. 2015; Salem et al. 2013). This new strategy has been used to treat contaminated soils (Hale et al. 2015; Beesley et al. 2010), essentially because they

are economically beneficial and environmental-friendly options (Khan et al. 2015; Tang et al. 2013).

Along with BC, biochar research has increased (Tang et al. 2013) and revealed its potential use for improving crop productivity, alleviating climate change, recycling agricultural wastes and remediating environments with contaminated with organic and inorganic xenobiotics (Oleszczuk et al. 2013; Tang et al. 2013; Yu et al. 2009; Zaifu et al. 2015). Biochar is the scientific term for the non-activated carbon-rich porous material, used as a soil amendment, produced by thermal degradation of a biomass (pyrolysis) in an environment without oxygen, preferably (Wang et al. 2013; Safaei Khorram et al. 2015; Ahmad et al. 2014; Nag et al. 2011; Sohi et al. 2010; Zhang et al. 2010). Its high poly aromatic-C structures' content has allowed the use of biochar as a soil conditioner to improve physical, chemical and biological soil properties (Ahmad et al. 2014; Beesley et al. 2010; Puga et al. 2015; Sohi et al. 2010; Oleszczuk et al. 2013). Besides, its high surface area and special structure (Tang et al. 2013) that allow its use as a remediation agent, biochar particles present two distinct phases responsible for different physical properties: the non-carbonized fraction – responsible for the partitioning/absorption -, and the carbonized fraction – where the adsorption occurs (Ahmad et al. 2014; Beesley et al. 2011).

In an agricultural context, when incorporating biochar in soil, besides improving soil properties, some effects on sorption and leaching of pollutants may occur (Larsbo et al. 2013) and, thus, affect the behaviour of organic and inorganic pollutants present (Jones et al. 2011; Wang et al. 2013; Sopeña et al. 2012; Zhang et al. 2010; Zaifu et al. 2015). This assumption can be discussed within the Equilibrium Partitioning Theory, where chemical toxicity to soil organisms is directly proportional to the amount of unbound chemicals, which are dissolved in the soil pore water (Di Toro et al. 1991; Belfroid et al. 1995). If chemicals are bound to organic carbon, they will not be available in the pore water and toxicity will be decreased. The reducing of negative impact of pesticides, like bioaccumulation, have been already reported to several groups of organisms (Oleszczuk et al. 2014; Oleszczuk et al. 2013). However, despite the recent worldwide interest on the possible effects biochar can have on the behaviour of these compounds in soil or on the improvement of soil water retention, pH, amongst others, few information is also available on the overall effects different biochars can induce in soil biota (Amaro et al. 2016; Lehmann et al. 2011).

Considering the potential uses of biochar to improve soil quality, the aim of this study was to evaluate whether biochar induced changes in a contaminated soil with dimethoate (Dimistar Progress®). For that, toxicity studies with soils contaminated with dimethoate alone and with two biochar amendments were carried out with *Folsomia candida* and *Brassica rapa*. Results will be further discussed in the light of the Equilibrium Partitioning Theory, looking at dimethoate concentrations in pore water.

2.2. Materials and Methods

2.2.1. Test soil and biochar characteristics

All tests were carried out in the LUFA 2.2 natural soil, commercialized by German Institute LUFA Speyer. This sandy loamy soil (76.1% sand, 16.2% silt, 7.7% clay) was chosen as a well-studied a natural soil. The physical-chemical characteristics are expressed in table 2.

Table 2. Physical and chemical characteristics of the LUFA 2.2 natural soil used in the ecotoxicological tests with collembolans and turnips.

Characteristics	Units	Values
Maximum WHC	g 100g ⁻¹	43.5 ± 2.8
Content in organic carbon	%	1.59 ± 0.13
Nitrogen content (% of N)	% of N	0.17 ± 0.01
pH value (0.01 M CaCl ₂)	-	5.4 ± 0.2
CEC	meq 100 g ⁻¹	9.7 ± 0.4

The biochar selected was obtained from Delinat Institute – Swiss Biochar in Switzerland. A mix of wood residues (from wood chip production) was pyrolysed at a temperature of 620 °C for 20 minutes. Physical and chemical properties are described in table 3.

Table 3. Physical and chemical properties of the selected biochar used in the reproduction test with *Folsomia candida* and in the germination test with *Brassica rapa*.

Properties	Units	Values
Density	kg m ⁻³	552
pH (CaCl ₂)	-	10.1
Moisture	w w ⁻¹	30
Ash (550 °C)	mg kg ⁻¹	5
Total C	w w ⁻¹	75
Total N	w w ⁻¹	1.8
Molar ratios		
• H:C	-	0.074
• O:C	-	0.041
Particle sizes		
• <0.1 mm		4
• 0.1-0.5 mm		25
• 0.5-2 mm	%	34
• >2 mm		37
Total contaminant contents		
• Sum of metals		171.27
• Sum of 16 PAHs		<0.48
• Sum of 7 indicator PCBs (dioxins)	mg kg ⁻¹	0.00176

2.2.2. Test chemical and soil spiking

Tests were carried out with the commercial formulation Dimistar® Progress from Cheminova A/S with 39% of dimethoate (as active substance), 48% of cyclohexanone, 8% of C9 aromatic hydrocarbon and 0.1-1% of maleic anhydride. Dimethoate (IUPAC name *O, O*-dimethyl *S*-[2-(methylamino)-2-oxoethyl]dithiosphosphate, CAS number 60-51-5 and empirical formula $C_5H_{12}NO_3PS_2$) is an OP compound characterized for inhibiting the enzyme AChE, a key enzyme in nervous system's functioning and responsible for the degradation of acetylcholine into acetyl and choline.

Two stock solutions of dimethoate in water of 17.84 mg L^{-1} (for the collembolan test) and 557.5 mg L^{-1} (for the plant test) were used as a starting point to achieve five different nominal concentrations for the ecotoxicity tests, ranging from 0.1 mg kg^{-1} to 1.6 mg kg^{-1} for the collembolan test, and ranging from 10 mg kg^{-1} to 50 mg kg^{-1} for the plant experiment. Soil, with and without biochar, was spiked considering the soil moisture content set to 60% of the WHC. At the beginning and end of the test, the pH of all treatments was measured (more information can be seen in tables B-SD and C-SD in the supplementary data section). Biochar was added to soil 96 hours before the beginning of the bioassays for stabilization. For these experiments, two applications were tested: 25 g kg^{-1} (BC25) and 50 g kg^{-1} (BC50) (equivalent to $2.5\% \text{ w/w}$ or 50 t ha^{-1} and $5\% \text{ w/w}$ or 100 t ha^{-1} , respectively). Dimethoate was then used to spike soil and soil amended with biochar in plastic boxes and then transferred to the glass test containers. The negative control was LUFA 2.2 soil moistened with ultrapure water (60% WHC), and controls for biochar application were also included (biochar only treatments (BC25 and BC50).

2.2.3. Test organisms

The laboratory cultures of *Folsomia candida* were kept in a controlled temperature room (constant temperature of $20 \pm 2 \text{ }^{\circ}\text{C}$ and photoperiod of 16h/8h (light/dark)) in plastic boxes that contained a mixture of activated carbon with plaster of Paris, in a proportion of 9:1, respectively. Once a week, granulated dried yeast was added to the culture as food source. To obtain synchronized culture organisms, 20 to 30 adults were placed in plastic boxes for two days to lay eggs in order to obtain juveniles with 10-12 days old, ready to be used in the tests.

Brassica rapa seeds were purchased from Carolina Biological Supply Company and maintained in bags in a climate room with controlled conditions (constant temperature of 20 ± 2 °C and photoperiod of 16h/8h (light/dark)).

2.2.4. *Folsomia candida* reproduction test

The reproduction test with the collembolan *F. candida* was carried out accordingly to the ISO guideline 11267 (ISO 1999).

Ten juveniles with 10-12 days old transferred from synchronized cultures to the glass test containers (5 cm diameter x 9.5 cm height), and kept in a test room for 28 days (Fig. 5), under similar conditions as those from laboratory cultures. Once a week, to allow aeration, test containers were opened and food and water were added (to replenish 60% WHC). At the end of the experiments, test containers were filled with water and gently stirred to promote the fluctuation of the survivals adults and juveniles. Finally, each replica was photographed to allow the counting of springtails, using the software Image J.



Figure 5. Glass test containers used for the *F. candida* reproduction test, performed based on the ISO procedure 11267.

2.2.5. *Brassica rapa* germination test

The effects of dimethoate on the emergence and growth of the rapid cycle turnip *Brassica rapa* was assessed using the ISO guideline 11269-2, with some adaptations as described by Santos et al (2011). The plant growth test room was kept at a constant temperature range of 22 ± 4 °C, photoperiod of 16h daylight and 8h dark and a light intensity of 7800 lux on the surface.

The plastic pots (11.5 cm top x 9 cm bottom x 10 cm height) used for this test were filled with 450 g of the test substrate – only soil (B0 test) and soil amended with biochar (B25 and B50) (Fig. 6). Ten seeds were distributed in the soil, at approximately 0.5 cm depth. As explained above, the soil moisture was adjusted to 60% WHC in the beginning of the test and maintained throughout the test by a water capillarity supply system. For this, a hole was open in the pot's bottom and a cotton wick introduced in direct contact with the substrate. Then the pot was placed on top a smaller one (9.5 cm top x 7.5 cm bottom x 4.5 cm height) with water for supply.

The seed's emergence was checked daily, and when 50% of the control seeds germinated, the experiment proceeded for more 14 days. At the end, all plants were cut right above the soil surface, measured and weighted (fresh weight). Plants dry weight was also recorded after a 48h period in an oven at 80 °C. Visual observation was carried out daily, to record any changes in plant colour that could highlight like necrosis.

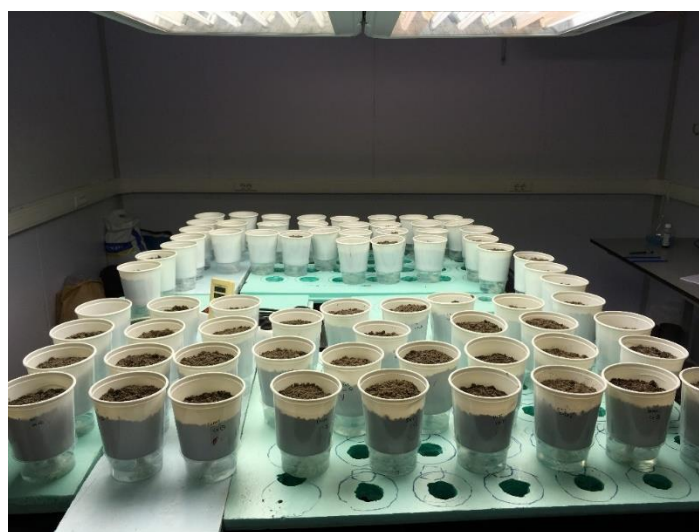


Figure 6. Plant germination test performed using the ISO protocol 11269-2, in a plant growth room.

2.2.6. Pore water extraction

Pore water extraction was carried out in soils spiked with dimethoate at concentrations of 0.1, 1.6, 10 and 50 mg kg⁻¹ in the absence or presence of biochar (B25 and B50) after 12h, 24h, 7 days, 14 days (the last two concentrations only) and 28 days (the first two concentrations only). For pore water extraction, 50 g of soil were saturated with ultrapure water and incubated for 48 hours equilibration. The saturated soil was then centrifuged for 90 minutes at a relative centrifugal force of 2860 g in an Eppendorf 5810R centrifuge. With a syringe and a cellulose nitrate filter (0.45 µm pore size), the supernatant was collected and stored at -18 °C until analysis, following the protocol described by Tourinho et al. (2013) with few adaptations.

2.2.7. Statistical analysis

The software SigmaPlot 12.5 from the company Systat Software Inc. was used for the statistical analysis of all the data obtained from these experiments.

Data was first tested for normality and homogeneity through the Kolmogorov-Smirnov test and Levene median test, respectively. To compare effects upon exposure, a One Way ANOVA was carried out, and dimethoate treatments were compared to the control, using a Dunnet test. Whenever data was not normally distributed, possible a square root transformation was performed to achieve these two assumptions. If those were not verified, Kruskal-Wallis test by ranks was used, followed by the Dunn's test to compare statistical differences between all treatments with the control group ($p < 0.05$). The values for the NOEC and LOEC were derived and reported. To calculate EC₅₀ and LC₅₀ values, a sigmoidal equation (logistic, 3 parameter) was used. To evaluate the interaction between the dimethoate concentration in soil and the biochar rate, a Two Way ANOVA was performed.

To depict statistical differences between the EC₅₀ calculated for length and fresh weight of the different tests, a chi-square test χ^2 was employed to the dose response curves output data of B0 vs B25 and B0 vs B50, and also B25 vs B50, using the equation below:

$$X^2 = df \times \ln \frac{r_2}{r_1}$$

Where df are the degrees of freedom, r_1 is the residual sum of squares for a EC_{50} value estimated from data from both curves that are to be compared and r_2 is the residual sum of squares for the two equations where each has its respective EC_{50} value. Differences were attained when $p < 0.05$.

2.3. Results

The results from dimethoate in the pore water extraction will not be reported in this thesis due to some delays in the chemical analysis.

2.3.1. *Folsomia candida* reproduction test

Different responses were observed for the survival and reproduction of *F. candida* exposed to biochar alone (B0) or with the two biochar rates used (B25 and B50) (Fig. 7 and 8).

There were no significant differences between the control treatments from the three tests – B0 vs B25, B0 vs B50 and B25 vs B50 - for the offspring production and survival (One Way ANOVA; $p > 0.05$). For the B0 test (Fig. 7 and 8), both parameters were affected in a dose-response manner (Dunnett test; $p < 0.05$). Toxicity was already observed at 0.4 mg kg^{-1} , with offspring production being significantly affected in about 38% compared to the control, about 88% in the concentration 0.8 mg kg^{-1} and about 99% at the highest concentration used (1.6 mg kg^{-1}). Survival was affected at the highest concentrations, with reductions on the number of adults of about 86% in 0.8 mg kg^{-1} and about 97% in 1.6 mg kg^{-1} concentration (more information can be seen in table A-SD in the supplementary data section). Two dose-response curves (Fig. 7 and 8) were obtained for both parameters and the EC_{50} for reproduction calculated as 0.45 mg kg^{-1} and the LC_{50} obtained for mortality 0.63 mg kg^{-1} (Table 4). NOEC and LOEC can be found in Table 4.

For the soils with dimethoate and biochar (B25 and B50 tests; Fig. 7 and 8), both survival and reproduction were not affected a dose-response manner ($p > 0.05$).

In the B25 test (Fig. 7 and 8), both the number of adults and the number of juveniles produced were not different between the treatments with dimethoate and the control ($p > 0.05$). The same pattern was observed for the B50 application rate, although a slight increase on the number of the juveniles produced was observed.

To see if offspring production and survival were affected by the interaction of both dimethoate concentration and biochar rate, a Two Way ANOVA was performed (Report on the Supplementary Data). The analysis of the data from the offspring production exhibited significant differences (Holm-Sidak test; $p < 0.05$) when comparing the biochar rates within the concentrations 0.4 mg kg^{-1} , 0.8 mg kg^{-1} and 1.6 mg kg^{-1} . No significant differences (Two Way ANOVA; $p > 0.05$) were obtained when comparing dimethoate concentrations within different rates of biochar. For survival, a similar pattern was found. Significant differences were obtained for the two concentration 0.8 mg kg^{-1} and 1.6 mg kg^{-1} when comparing the B0 vs B25 test and B0 vs B50 test (Holm-Sidak test; $p < 0.05$). Again, no significant differences (Two Way ANOVA; $p < 0.05$) were found between dimethoate concentrations and the control treatment within biochar rates.

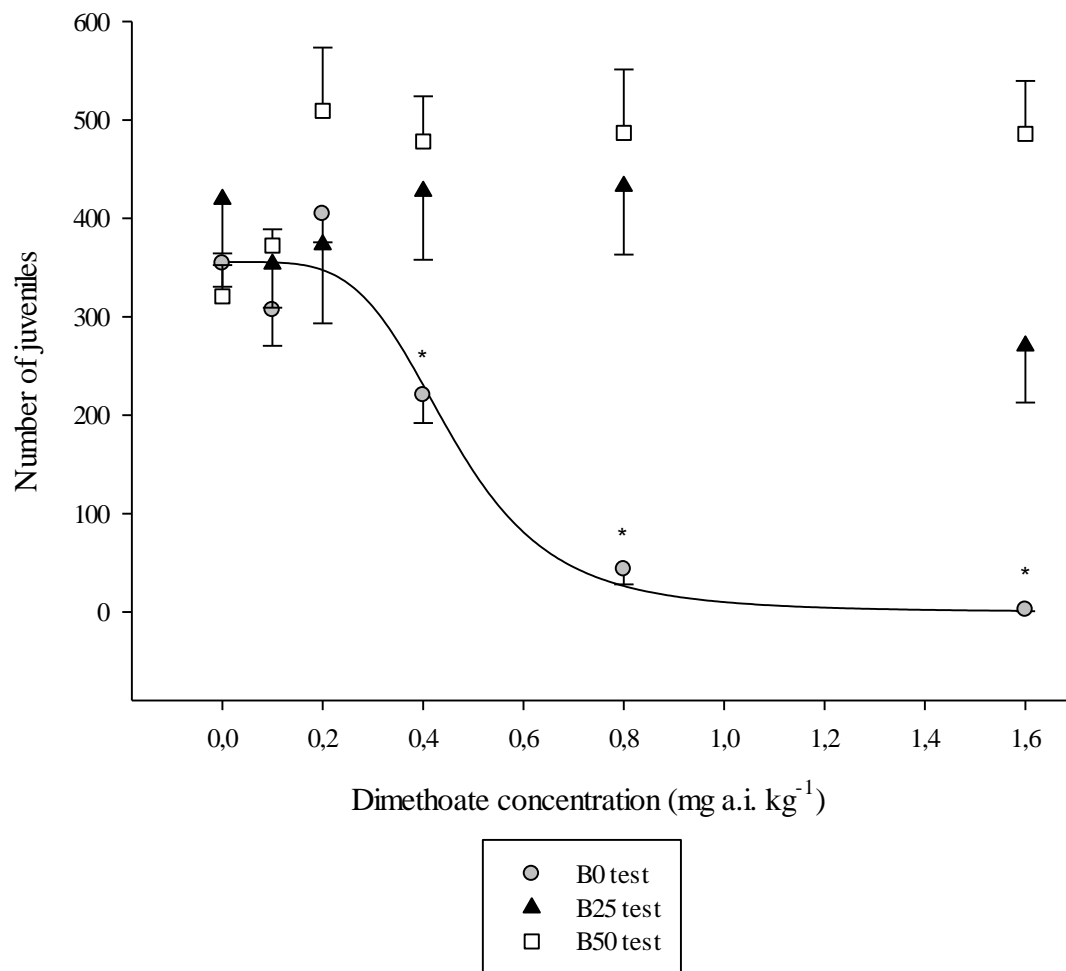


Figure 7. Number of juveniles produced by *Folsomia candida* exposed for 28 days to dimethoate in LUFA 2.2 natural soil, without biochar (B0 test) and with biochar (B25 and B50 test). The grey dots indicate the number of juveniles for the B0 test, the black triangles indicate the number of juveniles for the B25 test and the white squares represent the number of juveniles for the B50 test. All data are presented as mean values, with standard error bars (* when $p < 0.05$ Dunnett's test, indicating statistical differences when comparing contaminated treatments with the control treatment of the B0 test). The line represents data fit to obtain the EC_{50} for the B0 test.

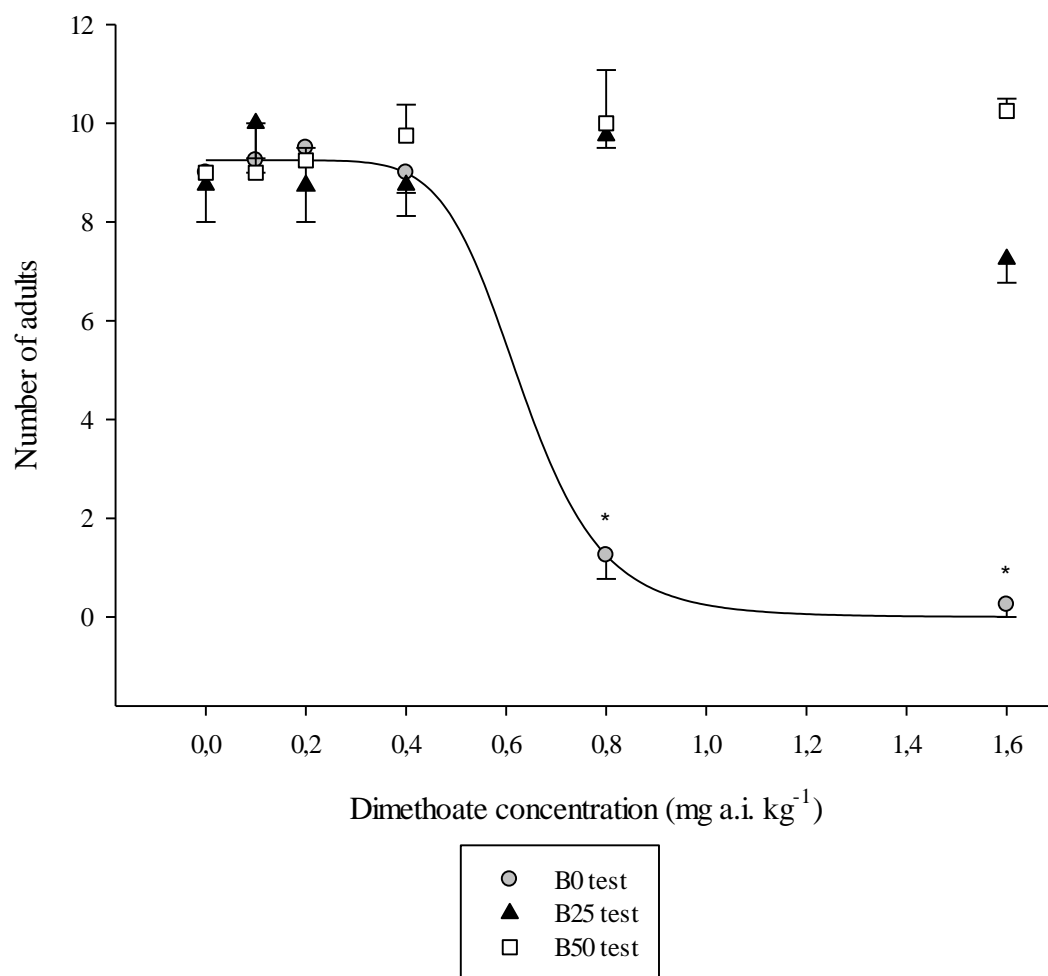


Figure 8. Number of survivors from *Folsomia candida* exposed to dimethoate for 28 days in LUFA 2.2 natural soil, without biochar (B0 test) and with biochar (B25 and B50 test). The grey dots indicate the number of adults retrieved at the end of the B0 test, the black triangles indicate the number of adults for the B25 test and the white squares for the B50 test. Data are expressed as mean values, with standard error bars (* when $p < 0.05$ Dunnett's test, indicating statistical differences when comparing contaminated treatments with the control treatment of the B0 test). The line represents data fit to obtain the LC_{50} for the B0 test.

Table 4. NOEC, LOEC, EC₅₀ and LC₅₀ values (mg a.i. kg⁻¹ soil) obtained for each test, according to the biochar rate (B0: no biochar, B25: 2.5% w/w biochar incorporated with soil and B50: 5% w/w of biochar incorporated with soil). Endpoints such as reproduction and mortality of *Folsomia candida* were recorded. Test occurred for 28 days in LUFA 2.2 natural soil in the following conditions of 16h daylight and 8h dark. NOEC and LOEC were derived from a One Way ANOVA followed by Dunnet test both endpoints. EC₅₀ and LC₅₀ were calculated through the use of a sigmoidal equation (logistic, 3 parameter).

Juveniles			
	B0 Test	B25 Test	B50 Test
NOEC	0.20 mg kg ⁻¹	> 1.60 mg kg ⁻¹	> 1.60 mg kg ⁻¹
LOEC	0.40 mg kg ⁻¹	> 1.60 mg kg ⁻¹	> 1.60 mg kg ⁻¹
EC ₅₀	0.45 ± 0.04 mg kg ⁻¹	> 1.60 mg kg ⁻¹	> 1.60 mg kg ⁻¹
Adults			
	B0 Test	B25 Test	B50 Test
NOEC	0.40 mg kg ⁻¹	> 1.60 mg kg ⁻¹	> 1.60 mg kg ⁻¹
LOEC	0.80 mg kg ⁻¹	> 1.60 mg kg ⁻¹	> 1.60 mg kg ⁻¹
LC ₅₀	0.63 ± 0.06 mg kg ⁻¹	> 1.60 mg kg ⁻¹	> 1.60 mg kg ⁻¹

2.3.2. *Brassica rapa* - germination test

Starting with the comparison of the different control groups from the three tests – B0 vs B25, B0 vs B50 and B25 vs B50 -, no significant differences were found for the endpoints shoot length and fresh weight (One Way ANOVA, $p > 0.05$). Looking at the sole application of dimethoate (B0 test) both shoot length and fresh weight were decreased in a dose-response manner (One Way ANOVA, Dunnet test; $p < 0.05$; Fig. 9 and 10). The first concentration applied, 10 mg kg⁻¹, caused already a significant decrease in the shoot length. The fresh weight was also affected by the pesticide, having a significant decrease of about 40% when comparing the control at 10 mg kg⁻¹. The EC₅₀, NOEC and LOECs are presented in table 5 for both parameters.

For the B25 test at 10 mg kg⁻¹ no deleterious effects were observed ($p > 0.05$) neither for the shoot length nor for the fresh weight of *B. rapa*, when compared to the control

treatment. Still, dimethoate induced a dose-response pattern for both parameters. For the B50 test, all concentrations showed a significant decrease in the shoot length ($p < 0.05$), with fresh weight presenting the same pattern observed for B25. The EC_{50} , NOEC and LOECs for B0, B25 and B50 are presented in Table 5 for both parameters.

In this test, a Two Way ANOVA was performed to evaluate the interaction between biochar rates within dimethoate concentration (Report on the Supplementary Data). For the shoot length, when comparing the differences between biochar rates (B0 *vs* B25 and B0 *vs* B50) within dimethoate concentrations, significant differences were obtained (Holmak-Sidak; $p < 0.05$). The B25 application rate affected this parameter at the concentrations 10 and 30 $mg\ kg^{-1}$ while the B50 application rate affected all concentrations. Significant differences were also obtained when comparing the different concentrations within different biochar rates (Holmak-Sidak; $p < 0.05$). For the fresh weight, significant differences were found when comparing different biochar rates (B0 *vs* B25 and B0 *vs* B50) within 10 and 30 $mg\ kg^{-1}$ (Holmak-Sidak; $p < 0.05$). When testing the different concentrations within different biochar rates, an equal pattern to the one for shoot length was observed.

Although there was still a dose response curve for both rates of biochar application, it could be observed that the toxicity induced by dimethoate was decreased with biochar application. Results from the comparison between the dose-response curves of all tests (X^2 test) for both endpoints, shoot length and fresh weight are presented in table 6 (more information can be seen in tables D-SD and E-SD in the supplementary data section).

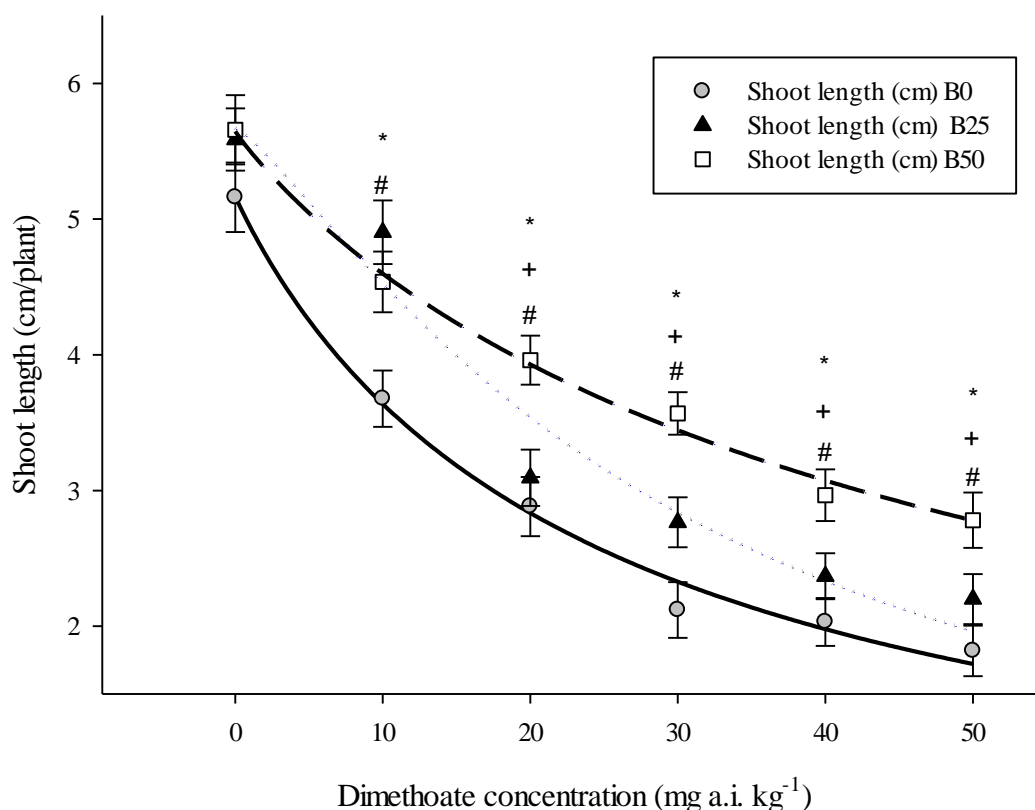


Figure 9. Dose-response curves for the shoot length (cm) of *Brassica rapa* after exposure to LUFA 2.2, a natural soil contaminated with dimethoate, without biochar (B0 test) and with biochar (B25 and B50 test). Grey dots indicate the shoot length for the B0 test, the black triangles indicate the shoot length for the B25 test and the white squares indicate the shoot length for the B50 test. Data are expressed as mean values with standard error bars (* when $p < 0.05$ Dunnett's test, indicating statistical differences when comparing contaminated treatments with the control treatment of the B0 test, + when $p < 0.05$ Dunn's test, indicating statistical differences when comparing contaminated treatments with the control of the B25 test and # when $p < 0.05$ Dunn's test, indicating statistical differences when comparing contaminated treatments with the control of the B50 test). Lines represent the sigmoidal equation (logistic, 3 parameter) used to obtain the EC_{50} for shoot length.

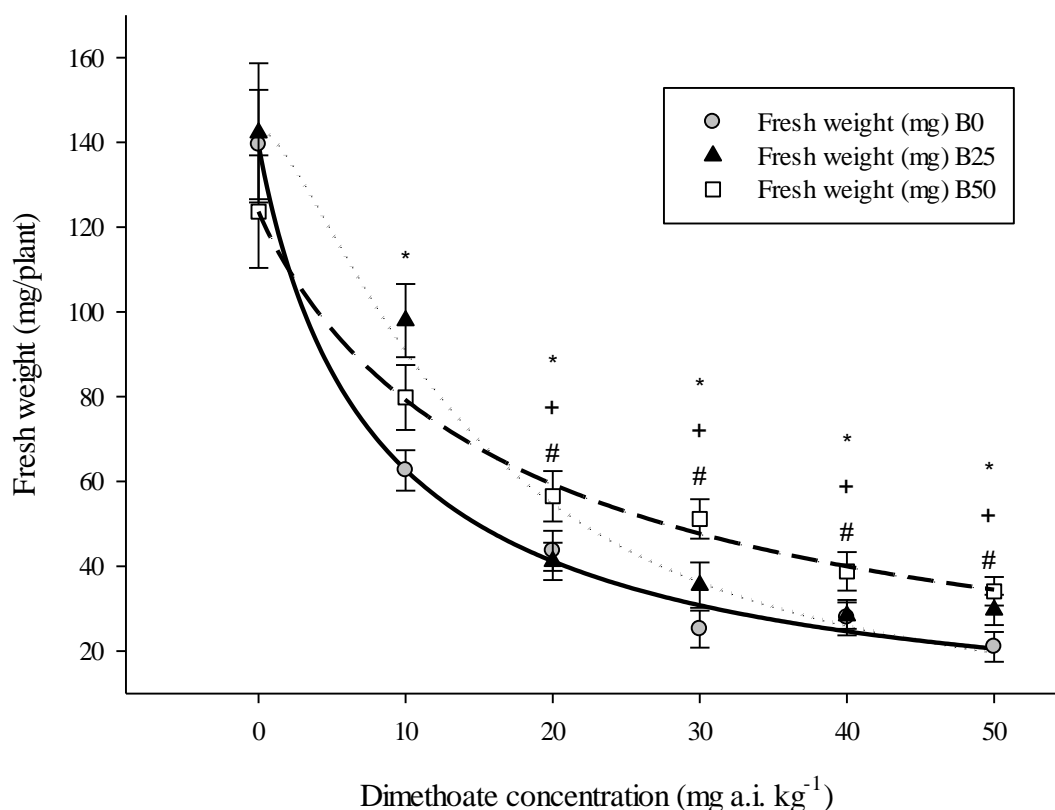


Figure 10. Dose-response curves for the fresh weight (mg) of *Brassica rapa* after exposure to LUFA 2.2, a natural soil contaminated with dimethoate, without biochar (B0 test) and with biochar (B25 and B50 test). Grey dots indicate the fresh weight for the B0 test, the black triangles indicate the fresh weight for the B25 test and the white squares indicate the fresh weight for the B50 test. Data are expressed as mean values, with standard error bars (* when $p < 0.05$ Dunnett's test, indicating statistical differences when comparing contaminated treatments with the control treatment of the B0 test, + when $p < 0.05$ Dunn's test, indicating statistical differences when comparing contaminated treatments with the control of the B25 test and # when $p < 0.05$ Dunn's test, indicating statistical differences when comparing contaminated treatments with the control of the B50 test). Lines represent the sigmoidal equation (logistic, 3 parameter) used to obtain the EC_{50} for fresh weight.

Table 5. NOEC, LOEC and EC₅₀ (mg a.i. kg⁻¹ soil) obtained for each test, according to the biochar rate (B0: no biochar, B25: 2.5% w/w of biochar incorporated with soil and B50: 5% w/w of biochar incorporated with soil). Parameters such as length and fresh weight of *Brassica rapa* were recorded. Test occurred for 14 days in a LUFA 2.2 natural soil. NOEC and LOEC were derived from a One Way ANOVA followed by Dunnet test for the parameter shoot length (cm) and followed by a Dunn's test for the parameter fresh weight (mg). EC₅₀ and LC₅₀ were calculated through the use of a sigmoidal equation (logistic, 3 parameter).

Shoot Length (cm)	B0 Test	B25 Test	B50 Test
NOEC	< 10 mg kg ⁻¹	10 mg kg ⁻¹	< 10 mg kg ⁻¹
LOEC	10 mg kg ⁻¹	20 mg kg ⁻¹	10 mg kg ⁻¹
EC ₅₀	24.43 ± 2.94 mg kg ⁻¹	28.72 ± 2.47 mg kg ⁻¹	44.04 ± 5.11 mg kg ⁻¹
Fresh weight (mg)	B0 Test	B25 Test	B50 Test
NOEC	< 10 mg kg ⁻¹	10 mg kg ⁻¹	10 mg kg ⁻¹
LOEC	10 mg kg ⁻¹	20 mg kg ⁻¹	20 mg kg ⁻¹
EC ₅₀	8.12 ± 2.00 mg kg ⁻¹	14.33 ± 1.99 mg kg ⁻¹	17.07 ± 3.14 mg kg ⁻¹

Table 6. Results of the Chi-square (X^2) test performed. EC₅₀ of all tests (B0, B25 and B5 test) for both parameters (shoot length and fresh weight) were compared. (* when $X^2 > 3.84$, statistical differences between treatments exist).

Shoot length	B0 vs B25	B0 vs B50	B25 vs B50
X^2 value	1.25	13.25 *	9.54 *
Fresh weight	B0 vs B25	B0 vs B50	B25 vs B50
X^2 value	5.06 *	6.19 *	0.53

2.4. Discussion

2.4.1. *Folsomia candida* and *Brassica rapa* as test models

According to Marks et al. (2014), many soil invertebrates are used to evaluate soil quality and ecotoxicity because of their high sensitivity to biotic and abiotic conditions, allowing the detection on trait changes. In addition, *F. candida* exposure route to contaminants in soil is mainly through the soil pore water and therefore it can be a good model to understand the sorption of dimethoate to biochar, as it is expected a decrease in toxicity due to the decrease of dimethoate concentrations in the pore water. This is also true for plants, like *B. rapa*, although the toxicity pattern upon biochar application did not show so much evidences on the complete immobilization of dimethoate. As it is known, species traits, along with the media type are mainly related to the bioavailable fraction for uptake during exposure. In this case, species traits are different between the insect and the plant species, except for the soil pore water exposure route. In addition, although the soil type was the same, the exposures differed in terms of concentration ranges (which will be discussed below).

Although results from dimethoate chemical analysis in pore water are not available yet, we expect lower concentration in the pore water upon biochar amendment.

2.4.2. Dimethoate exposure

In the B0 test with *F. candida*, both the number of juveniles and the number of adults decreased with the pesticide dose increasing, with the offspring production significantly affected at 0.4 mg kg^{-1} . Although this concentration is ecological relevant as it represents a similar recommended field dose for this insecticide application (Santos et al. 2010), it has shown to induce hazard to soil organisms, and therefore risk may exist upon dimethoate application. In the study of Ferreira et al. (2015), it was observed that although this pesticide concentration has a high degradation rate ($0.32/\text{d}$), effects at the nervous system of the terrestrial isopod *Porcellionides pruinosus* were detected just after 48h of exposure. Martikainen (1996) studied the dimethoate toxicity to the earthworm *Aporrectodea caliginosa tuberculata*, to the collembolan *Folsomia candida* and to the

enchytraeid *Emchytraeus crypticus/variatus* in different soils (artificial, clayey and hummus soil). The effects of dimethoate on the earthworm *A. caliginosa tuberculata* was assessed through survival and biomass, which had different results, depending on the soil type. For the survival, this endpoint was more affected in the clayey soil (40 mg kg⁻¹), followed by the artificial (56 mg kg⁻¹) and then the hummus soil (65 mg kg⁻¹). For biomass, the same trend was found, however at lowest concentrations. The enchytraeid *E. crypticus/variatus* were less affected by dimethoate than collembolans. Differences were found between the number of adult surviving in the different soils and not between concentrations and the same was found to the number of juveniles. The reproduction of collembolan *F. candida* had different IC₅₀ values, according to the soil type: 6.3 mg kg⁻¹ in the artificial soil, 3.8 mg kg⁻¹ for the clayey soil and 5.5 mg kg⁻¹ for the hummus sandy soil. Loureiro et al. (2005) tested the effect of dimethoate in the earthworm *Eisenia Andrei* and in the isopod *Porcellionides pruinosus*. Avoidance behaviour showed that earthworms exposed to dimethoate contaminated soil retrained an EC₅₀ of 50.07 mg kg⁻¹ and when isopods were exposed individually an EC₅₀ of 39.43 mg kg⁻¹ was calculated while upon group exposure an EC₅₀ of 28.67 mg kg⁻¹ was achieved. Another study, by De Silva and Amarasinghe (2008) evaluated the effect of dimethoate on the common compost worm *Eisenia andrei*, using the OECD soil and a natural soil as substrates. The EC₅₀ values reached were 24.06 mg kg⁻¹ for the OECD soil and 13.76 mg kg⁻¹ for the natural soil, showing that the soil type can influence the toxicity of compounds to organisms.

In addition, in the present study was observed the attempt of collembolans to climb the glass vessels, trying to avoid the contaminated soil. As mentioned before collembolans can detect toxic compounds by using their olfactory sense, with the detection of chemicals by the PAO.

The effect of dimethoate to *F. candida* was already studied, by several authors (e.g. Martikainen (1996) (as discussed above), Chowdbury et al. (2001), Krogh (2008), Santos et al. (2010) and Amorim et al. (2012)). Chowdbury et al. (2001) exposed *F. candida* in Petri dishes with leaves from different sources (barley, maize, rape, cabbage, tomato, pear, sugarcane, wheat, orange and dwarf bean) that were sprayed before the with exposure dimethoate. Their results showed that the transfer of the contaminant to the collembolan organism depended not only on the properties of the pesticide but also on the substrate characteristics. The mortality of *F. candida* increased with the pesticide

dose, having almost 100% mortality on most surfaces. In this study, deltamethrin was also studied and a different response was observed when *F. candida* was exposed. The leaf surface characteristics and the pesticide physicochemical properties were effective in the availability of this compound to *F. candida*, which reflected in different toxicity to the collembolan. Krogh (2008) tested the effect of dimethoate as pure compound on the reproduction and mortality of *F. candida* and obtained an EC₅₀ of 1.65 mg kg⁻¹ and a LC₅₀ of 2.1 mg kg⁻¹. On the other hand, Santos et al. (2010) used a commercial formulation with dimethoate as active ingredient, and obtained an EC₅₀ of 0.37 mg kg⁻¹. Amorim et al. (2012) used dimethoate also as pure compound and obtained an EC₅₀ of 1.6 mg kg⁻¹. The EC₅₀ obtained in the present study (0.45 mg kg⁻¹) is similar to the one described by Santos et al. (2010), which used also a dimethoate formulation (AGROR®). In the same study, Santos et al. (2011) studied the effects of commercial formulation on *B. rapa* and *Triticum aestivum* and obtained less deleterious effects, when comparing to this study. *T. aestivum* had an EC₅₀ values for shoot length and fresh weight of 19 mg kg⁻¹ and 11 mg kg⁻¹, respectively, while *B. rapa* had an EC₅₀ of 36 mg kg⁻¹ for the shoot length and an EC₅₀ of 29 mg kg⁻¹ for the fresh weight. The fresh weight was more affected than the shoot length in both plants and the EC₅₀ values obtained in each test were not very similar, indicating that even plant species can have different sensitivity responses to the pesticide. Comparing results and the dimethoate concentrations used, collembolans shown to be more sensitive than the rapid cycle turnips. Although this could be explained by the different excipients present in both formulations, the toxicity test results with *F. candida* did not show the same pattern. One of the solvents, cyclohexanone, which is present at higher concentration in the formulation used in the present study, could be one of the reasons why plants were more affected, increasing possible the presence of the compound in pore water.

This highlights commercial dimethoate formulations seem to induce higher toxicity than the pure compound, and an underestimation of hazard is carried out when only the active ingredient is tested.

2.4.3. Biochar joint application with dimethoate in soil

According to Tang (2013), biochar is known to be an efficient sorbent for organic and inorganic contaminants. The major purpose of this remediation method is to reduce the pollutant's bioavailability, by adsorbing and immobilizing chemicals onto the biochar particles (Hale et al. 2015).

To our knowledge, no study about the biochar's effect on the adsorption of dimethoate was done. For these reasons, and because dimethoate is a well-known and worldwide used pesticide, we decided to explore this situation. Two non-target organisms were used to check the bioavailability of dimethoate and its effect on endpoints like the offspring production and adult survival for collembolans, shoot length and fresh weight for turnips. By the results obtained from the bioassays, it is possible to state that biochar can affect the behaviour of dimethoate in soil, mainly because of its capacity to adsorb it onto its surface, making it less bioavailable for the tested organisms.

Two biochar rates were applied in soil previously to dimethoate contamination, in order to assess its potential to immobilize dimethoate onto its particles surface. This assumption was tested on the Equilibrium Partitioning theory, which states that the concentration of a chemical in an organisms is determined by its uptake through the water phase. This theory was described for sediment organisms (Di Toro et al. 1991; OECD 1992) and also for earthworms (Belfroid et al. 1995). Therefore, what is extracted in the soil pore water will be considered the bioavailable fraction, which will be responsible by deleterious effects observed in collembolan and plants.

The B25 test, with 25 g kg⁻¹ of biochar, showed that this small rate of biochar can have some effect on the adsorption and immobilization of dimethoate, making it less bioavailable for the collembolans. This can be assumed due to the absence of dose-response curves for both endpoints and due to the low toxicity obtained. On the B50 test, with 50 g kg⁻¹ of biochar, results were similar, with even an increase in juveniles' production (although not statistically different from control). The dimethoate immobilization by biochar can be explained by the Two Way ANOVA output. This analysis tested, at first, the biochar effects within all concentrations, and its results suggest that biochar could have immobilized and decreased dimethoate effects on offspring production and survival in some concentrations because differences were found – 0.4, 0.8

and 1.6 mg kg⁻¹ for offspring production and 0.8 and 1.6 mg kg⁻¹ for survival. When comparing different concentrations with the control within different biochar rates, no differences between concentrations were found in the B25 and B50, which explains that dimethoate did not affected offspring production and survival in soils amended with biochar, suggesting biochar efficiency on the retention of dimethoate.

In the present work the interaction between collembolans and biochar particles were not deeply assessed, besides reproductive and survival parameters, which did not differ from exposure to LUFA 2.2 soil when both biochar application rates were tested. In some cases, when organisms fed on biochar particle, the remediation aim can be reversed and even act as a contaminant carrier. Salem et al. (2013) verified that the collembola *Coecobrya tenebricosa* and *Folsomia fimetaria* could fed on biochar particles, and use them as good food source to complete their life cycle. These species only ingested hydrochar (type of char produced by hydrothermal carbonization (Salem et al. 2013) when it was the only food choice. In the present study, while this may happened, it did not represented an extra source for dimethoate exposure.

Along with earthworm's avoidance tests, plant germination tests have been used to characterize biochar exposure before their application in the field (Domene et al. 2015).

In the present study, and using a similar approach as the one used for collembolans, it was observed that both biochar application reduced dimethoate toxicity to *B. rapa*, but not totally as observed for collembolan.

The presence of 2.5% w/w of biochar (B25 test) showed a decrease on the toxicity, although there was still a dose-response pattern on the dimethoate exposure. When adding 5% w/w, the endpoint fresh weight was the one that most benefited from this application. Although significant differences between treatments and the control were found in all the concentrations for the shoot length, we can see a less marked effect. The shoot length mean value increased, for all treatments, from the B25 test to the B50 test. A similar pattern was observed for the fresh weight; the first concentration was not considered significantly different from the control and the remaining concentrations were positively affected with the increase of the biochar rate. In this test, the Two Way ANOVA results can also explain biochar effect in dimethoate immobilization. When comparing contaminated soils with dimethoate with and without biochar, within concentrations, it

was possible to see that 5% w/w of biochar influenced dimethoate exposure, when comparing with the test that had no biochar. However, unlike what happened for the *F. candida* test, biochar effects were not enough to cause differences between dimethoate concentrations and the control treatment, which means that the rates used were not strong enough to immobilize dimethoate (at the concentrations used) in a way that no effects were attained.

Looking for the EC₅₀ values of this plant test (Table 5), it was possible to state that the increase of the biochar rate lead to an increase of the EC₅₀ value. This pattern may have been caused by the biochar sorption of dimethoate to biochar particles.

Several studies related the sorption effect of biochar to organic and inorganic contaminants. Yang et al. (2006) tested the effect of a wheat straw char on the sorption of diuron and found that only 1% of char was capable of remediating more than 86%, resulting in the increase of the fresh weight and of the survival rating of *Echinochloa crus-galli*, a barnyardgrass. With these results, Yang et al. (2006) proved that the bioavailability to soil microorganisms was reduced and the biodegradation, as well as the efficacy of the herbicide, were also reduced. In another study, Yu et al. (2009) assessed the effect of wood chip biochars on the remediation of two insecticides, chlorpyrifos and carbofuran, and they found that this amendment caused a decrease in the dissipation of its residues. Their results included higher biomass production of spring onions, when cultivated in soils mixed with biochars, and lower root concentration factor (RCF) of both pesticides with the increase of biochar rates in soils, showing that the biochar used reduced the bioavailability, and therefore was effective in reducing the uptake. Another study, by Zhang et al. (2010) exhibited the biochar ability to remediate phenanthrene, a hydrophobic polycyclic aromatic hydrocarbon, by allowing a decrease on its bioavailability to microbial degradation, plant uptake and non-target organisms. Oleszczuk et al. (2014) showed that different effects can be induced by biochar depending in the type of organisms tested and the pesticide tested too. Although the enzymatic activity was protected and increased by biochar, organisms like *Vibrio fischeri* were affected negatively by biochar and *Lepidium sativum* had different responses for the elutriates from soils (toxic effect) and for the solid phase (positive effect). Also, 2,4-D was considered more toxic with the increase of the dose of biochar.

Typical agronomic application rates of commercial biochar were also shown to influence simazine behaviour, decreasing its availability and degradation, and also reducing its leaching (Jones et al. 2011). Moreover, it was suggested that sorption to biochar prevented pesticide mineralization, limiting its availability to the soil microbial community. By suggested by Jones et al. (2011), comparisons between chemicals with low K_{ow} (octanol/water partition coefficient) can be done, which it is the case of dimethoate. Regarding herbicide persistence, Nag et al. (2011) measured the persistence of atrazine and trifluralin in soils and confirmed biochar's role in sorption and immobilization of these herbicides. Still on the herbicide persistence, Sopena et al. (2012) found that 1% and 2% of wood charcoal biochar application reduced the dissipation rate and degradation of isoproturon (IPU) and the IPU's sorption increased with the increasing application of biochar. With the decrease in the IPU concentration in the aqueous phases, the mineralization was affected, the transformation limited and the contaminant was less accessible to microorganisms. Si et al. (2011) obtained also a reduction on IPU's leaching caused by sorption to a charcoal amendment, resulting in a reduced microbial degradation; however, its capacity to control weed was not affected. Regarding inorganic contaminants, Puga et al. (2015) studied the biochar effect in a mine contaminated soil and showed that the available concentrations of some metals decreased, also in the pore water.

The differences between tests with and without biochar can represent the decrease of pesticides' root uptake by the plants and, respectively, decrease of toxic effects and contamination of the food chain. This has been suggested and proven by many studies for several types of pollutants such as heavy metals (Tang et al. 2013), pesticides and other pest controls (Graber & Kookana 2015). Tang et al. (2013) reported that there are evidences on the potential effectiveness of biochar in removing these contaminants from aqueous solutions and soils; and with them immobilized, less impacts will impair the environment. Enhancing sorption by increasing biochar rates resulted in a reduced bioavailability for this plant. However, for *B. rapa* test, even though toxicity decreased, we had better results in terms of remediation with *F. candida*. Although no chemical analysis is still available for pore water, results will be discussed considering the hypothesis that dimethoate will be decreased in pore water due to biochar application. This decrease may not be dose dependent, and it is expected that at higher dimethoate concentrations, biochar ability to immobilize the pesticide will be decreased when

compared to lower doses. This may be related to the number of sites available in biochar particles to act a sorbent.

Considering all this, biochar amendments in soils where dimethoate application will occur can protect collembolan populations and also decrease the availability of dimethoate to plants. Considering that the application advised dose is lower than the one used in this study for plants, if overdosing applications occurs, then biochar will reduce the potential bioavailability to plants, which is beneficial for human health.

2.5. Conclusion

As previously showed by other studies, dimethoate can be toxic to non-target organisms, such as the collembola *Folsomia candida* and the plant *Brassica rapa*. The aim of this study was to evaluate if biochar could induce changes on the toxicity of dimethoate to these organisms, by decreasing its bioavailability. One test without biochar (B0 test) was made to see how dimethoate would affect the mortality and reproduction of *F. candida* and germination, shoot length and fresh weight of *B. rapa*. Then, two rates of biochar were amended with soil (B25 and B50 tests) to see how these endpoints would react with the biochar inclusion. After biochar amendment, the toxicity of dimethoate to the non-target organisms decreased and it was the most effective in the *F. candida* test, since no dose-response curve was obtained for the treatments with biochar, nor significant deleterious effects. However, despite biochar had not shown an evident effect on the pesticide immobilization in plants because dose-response curves were still clearly obtained in the treatments with biochar, speculations about biochar sorption can be made, due to a lower toxicity observed in the endpoints studied in biochar tests. Both results are an example of biochar capacity on sorbing contaminants, allowing the decrease of the bioavailability and, therefore, dimethoate toxicity to these organisms, by immobilization it into its surface.

2.6. References

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CHAPTER III

FINAL CONCLUSIONS AND REMARKS

3. Final conclusions and remarks

The interest in biochar is connected to *Terra Preta do Índio* in Amazon and, ever since then, the effects of biochar in the restructuration of agricultural and industrial soils – through the increasing of water and nutrient retention, improving of soil fertility and sequestration of carbon – have been studied by the scientific community (Verheijen et al. 2010; Ahmad et al. 2014; Cabrera et al. 2014; Lehmann et al. 2011).

Recently, biochar has started to show its potential as an environmental sorbent for organic and inorganic contaminants in the environment (Ahmad et al. 2014; Beesley & Marmiroli 2011; Beesley et al. 2010; Li et al. 2013; Cabrera et al. 2014; van Der Kooij et al. 1991; Koltowski & Oleszczuk 2016; Janus et al. 2015), essentially due to its large surface area and cation exchange capacity (Beesley et al. 2011; Cederlund et al. 2016). The aim of this remediation is the reduction of pollutant risks to soils, sediments, water or receptor organisms (Beesley et al. 2011; Gomez-Eyles et al. 2011; Graber & Kookana 2015). Several studies showed its effects on the sorption capacity of organic contaminants (Cabrera et al. 2014; Cederlund et al. 2016; Chen & Chen 2009; Gomez-Eyles et al. 2011; Jones et al. 2011) and inorganic contaminants (Beesley & Marmiroli 2011; Beesley et al. 2010; Puga et al. 2015; Rehman et al. 2016). The remediation process will reduce the bioavailable fraction of the active ingredient, making it less toxic for the soil biota, and preventing further risks.

The main goal of this study was to evaluate the biochar's effect on the bioavailability and dimethoate toxicity. Two non-target organisms - the collembolan *F. candida* and the plant *B. rapa* - and two application rates of biochar – 2.5% (w/w) and 5% (w/w) - were used in this evaluation. This conclusion will be divided into three sub-sections. Conclusions about the test models chosen, the dimethoate exposure and the biochar effect in the bioavailability and toxicity of dimethoate to soil will be addressed.

3.1. Test models chosen for this study

Some terrestrial organisms, such as collembolans, and terrestrial plants, are used in toxicity tests due to the reliability of standardized protocols provided by ISO and OECD,

allowing the comparison between studies and, thus, the information's globalization. As representative species, any effects to *F. candida* and *B. rapa* would reveal what may occur in the soil ecosystem, whenever exposed to a hazardous agent. Both organisms can be affected with contaminants available in the soil's pore water because this is their route of exposure to acquire nutrients and water. Dimethoate is an OP hydrophilic compound, so the main interest was to use organisms whose way of exposure was the soil's pore water. According to the results obtained, both organisms were good test models in this study as both displayed dimethoate effects and biochar efficiency in the remediation of soils contaminated by the pesticide.

3.2. Dimethoate exposure

In this study, the active ingredient used was within a commercial formulation. Comparing the results obtained in this study with others from different studies, it was confirmed that the toxicity obtained from the commercial formulation was higher than the pure active ingredient. This may have happened due to other ingredients used in commercial formulations that help on the active ingredient efficacy towards the target organism. These results highlight the importance of choosing a commercial formulation instead of a pure compound, since an underestimation of the hazard may occur. However, despite the choice of commercial formulation being the most ecologically relevant, one must take into account that different commercial formulations may cause different levels of toxicity to organisms, as there could be different ingredients with simultaneously different concentrations. The pore water's chemical analysis is an important component that provides information on the bioavailable fraction capable of affecting organisms. By analysing the soil as a whole, this information would not be obtained. Although this information on dimethoate concentrations in pore water is not yet available for this thesis, we highlight the expected as a final conclusion for the results obtained, i.e., immobilization of dimethoate by biochar will lead to low concentration of dimethoate in soil pore water.

3.3. Biochar effect on the bioavailability of dimethoate to the test models

Biochar affected the behaviour of dimethoate in soil by making it less bioavailable for the tested organisms. Both test models can be affected by the bioavailable fraction of dimethoate in the soil pore water due to the common route of exposure for both organisms, and therefore, a chemical analysis to this fraction may help assess the dimethoate concentration, which is presented for uptake by collembolans and plants. The results obtained in both works showed biochar affected in decreasing the bioavailable fraction for uptake, like referred above. Even though the biochar's impact on the sorption of the bioavailable fraction was different between model organisms, it was proved by the absence of dose-response curves for both endpoints tested in *F. candida* reproduction test and by the increase of the EC₅₀, NOEC and LOEC values for the *B. rapa* germination test that biochar particles immobilize dimethoate, making it less bioavailable. These results show biochar's potential in remediating organic and inorganic compounds, allowing the treatment of contaminated soils. The application of biochar in soils can help in the retention of chemical compounds which are prejudicial for the ecosystem, besides all the other functions responsible for the restructuration of agricultural and industrial soils.

This study may open doors for future research in biochar and more data is required before biochar is implemented in remediation plans (Verheijen et al. 2010; Oleszczuk et al. 2014) More studies with different types of biochar, different toxic compounds, as well as their mixtures, and model organisms with different exposure routes (e.g. via soil particles, organic matter) should be carried out to decrease the knowledge gap that exists in this research area.

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Supplementary data

Table A. Mean number of the number of juveniles and the number of adults obtained for different treatments in the *Folsomia candida* reproduction test. B0 represents the test without biochar amended with soil, B25 represents the test with 25 g kg⁻¹ of biochar amended with soil and B50 represents the test with 50 g kg⁻¹ amended with soil.

B0 test						
Treatments	0 mg kg ⁻¹	0.1 mg kg ⁻¹	0.2 mg kg ⁻¹	0.4 mg kg ⁻¹	0.8 mg kg ⁻¹	1.6 mg kg ⁻¹
Mean						
number of juveniles	354	307	405	220	44	3
Mean						
number of adults	9	9	9	9	1	0
B25 test						
Treatments	0 mg kg ⁻¹	0.1 mg kg ⁻¹	0.2 mg kg ⁻¹	0.4 mg kg ⁻¹	0.8 mg kg ⁻¹	1.6 mg kg ⁻¹
Mean						
number of juveniles	420	354	374	428	433	271
Mean						
number of adults	9	10	9	9	10	7
B50 test						
Treatments	0 mg kg ⁻¹	0.1 mg kg ⁻¹	0.2 mg kg ⁻¹	0.4 mg kg ⁻¹	0.8 mg kg ⁻¹	1.6 mg kg ⁻¹
Mean						
number of juveniles	321	373	509	478	487	486
Mean						
number of adults	7	9	9	9	9	10

Table B. Values of soil pH obtained in the beginning and at the end of the *Folsomia candida* reproduction test. B0 test corresponds to the test without biochar amended with soil, B25 corresponds to the test with 25 g kg⁻¹ of biochar amended with soil and B50 corresponds to the test with 50 g kg⁻¹ of biochar amended with soil.

	Concentrations	Initial pH	Final pH
B0 test	0 mg kg ⁻¹	6.71	6.59
	0.1 mg kg ⁻¹	7.00	6.57
	0.2 mg kg ⁻¹	7.02	6.57
	0.4 mg kg ⁻¹	7.01	6.56
	0.8 mg kg ⁻¹	6.90	6.55
	1.6 mg kg ⁻¹	6.80	6.57
B25 test	0 mg kg ⁻¹	6.72	6.60
	0.1 mg kg ⁻¹	6.93	6.62
	0.2 mg kg ⁻¹	6.91	6.60
	0.4 mg kg ⁻¹	6.97	6.59
	0.8 mg kg ⁻¹	6.79	6.62
	1.6 mg kg ⁻¹	6.78	6.69
B50 test	0 mg kg ⁻¹	6.79	6.80
	0.1 mg kg ⁻¹	6.92	6.79
	0.2 mg kg ⁻¹	6.95	6.87
	0.4 mg kg ⁻¹	6.92	6.80
	0.8 mg kg ⁻¹	6.87	6.92
	1.6 mg kg ⁻¹	6.84	6.92

Table C. Values of soil pH obtained in the beginning and in the end of the *Brassica rapa* germination test. B0 test corresponds to the test without biochar amended with soil, B25 corresponds to the test with 25 g kg⁻¹ of biochar amended with soil and B50 corresponds to the test with 50 g kg⁻¹ of biochar amended with soil.

	Concentration	Initial pH	Final pH
B0 test	0 mg kg ⁻¹	6.26	7.46
	10 mg kg ⁻¹	6.36	7.31
	20 mg kg ⁻¹	6.34	7.18
	30 mg kg ⁻¹	6.35	7.02
	40 mg kg ⁻¹	6.35	6.93
	50 mg kg ⁻¹	6.38	6.67
B25 test	0 mg kg ⁻¹	6.30	7.38
	10 mg kg ⁻¹	6.33	7.24
	20 mg kg ⁻¹	6.33	7.16
	30 mg kg ⁻¹	6.34	6.96
	40 mg kg ⁻¹	6.33	6.93
	50 mg kg ⁻¹	6.39	6.68
B50 test	0 mg kg ⁻¹	6.33	7.41
	10 mg kg ⁻¹	6.35	7.24
	20 mg kg ⁻¹	6.34	7.16
	30 mg kg ⁻¹	6.34	6.94
	40 mg kg ⁻¹	6.38	6.92
	50 mg kg ⁻¹	6.36	6.89

Report of the Two Way Analysis of Variance

1. *Folsomia candida* reproduction test

a. Endpoint: Offspring production

Two Way Analysis of Variance

Comparisons for factor: **Biochar within 0**

Comparison	Diff of Means	t	P	P<0,05
0,000 vs. 2,500	87,833	1,559	0,234	No
0,000 vs. 5,000	16,000	0,284	0,778	No

Comparisons for factor: **Biochar within 0,1 mg kg⁻¹**

Comparison	Diff of Means	t	P	P<0,05
0,000 vs. 2,500	39,000	0,400	0,905	No
0,000 vs. 5,000	16,500	0,169	0,866	No

Comparisons for factor: **Biochar within 0,2 mg kg⁻¹**

Comparison	Diff of Means	t	P	P<0,05
0,000 vs. 5,000	104,750	1,518	0,252	No
0,000 vs. 2,500	31,000	0,449	0,655	No

Comparisons for factor: **Biochar within 0,4 mg kg⁻¹**

Comparison	Diff of Means	t	P	P<0,05
0,000 vs. 5,000	258,000	3,738	<0,001	Yes
0,000 vs. 2,500	207,500	3,006	0,004	Yes

Comparisons for factor: **Biochar within 0,8 mg kg⁻¹**

Comparison	Diff of Means	t	P	P<0,05
0,000 vs. 5,000	443,500	6,426	<0,001	Yes
0,000 vs. 2,500	389,250	5,640	<0,001	Yes

Comparisons for factor: **Biochar within 1,6 mg kg⁻¹**

Comparison	Diff of Means	t	P	P<0,05
0,000 vs. 5,000	483,500	7,005	<0,001	Yes
0,000 vs. 2,500	268,000	3,883	<0,001	Yes

Comparisons for factor: **Dimethoate within 0%**

Comparison	Diff of Means	t	P	P<0,05
0,000 vs. 1,600	321,500	5,103	<0,001	Yes
0,000 vs. 0,800	280,500	4,452	<0,001	Yes
0,000 vs. 0,400	103,750	1,647	0,284	No
0,000 vs. 0,200	80,500	1,278	0,371	No
0,000 vs. 0,100	26,000	0,326	0,746	No

Comparisons for factor: **Dimethoate within 2,5%**

Comparison	Diff of Means	t	P	P<0,05
0,000 vs. 1,600	141,333	2,243	0,137	No
0,000 vs. 0,100	100,833	1,265	0,613	No
0,000 vs. 0,200	38,333	0,608	0,906	No
0,000 vs. 0,800	20,917	0,332	0,933	No
0,000 vs. 0,400	15,917	0,253	0,802	No

Comparisons for factor: **Dimethoate within 5%**

Comparison	Diff of Means	t	P	P<0,05
0,000 vs. 0,200	169,250	2,686	0,047	Yes
0,000 vs. 0,800	147,000	2,333	0,090	No
0,000 vs. 1,600	146,000	2,317	0,071	No
0,000 vs. 0,400	138,250	2,194	0,064	No
0,000 vs. 0,100	26,500	0,333	0,741	No

b. Endpoint: Survival

Two Way Analysis of Variance

Comparisons for factor: **Biochar within 0 mg kg⁻¹**

Comparison	Diff of Means	t	P	P<0,05
0,000 vs. 5,000	0,0686	0,413	0,899	No
0,000 vs. 2,500	0,0438	0,276	0,783	No

Comparisons for factor: **Biochar within 0,1 mg kg⁻¹**

Comparison	Diff of Means	t	P	P<0,05
0,000 vs. 5,000	0,0651	0,237	0,965	No
0,000 vs. 2,500	8,882E-016	3,234E-015	1,000	No

Comparisons for factor: **Biochar within 0,2 mg kg⁻¹**

Comparison	Diff of Means	t	P	P<0,05
0,000 vs. 2,500	0,123	0,636	0,777	No
0,000 vs. 5,000	0,0326	0,168	0,867	No

Comparisons for factor: **Biochar within 0,4 mg kg⁻¹**

Comparison	Diff of Means	t	P	P<0,05
0,000 vs. 5,000	0,120	0,617	0,789	No
0,000 vs. 2,500	0,0457	0,235	0,815	No

Comparisons for factor: **Biochar within 0,8 mg kg⁻¹**

Comparison	Diff of Means	t	P	P<0,05
0,000 vs. 5,000	2,192	11,289	<0,001	Yes
0,000 vs. 2,500	2,165	11,148	<0,001	Yes

Comparisons for factor: **Biochar within 1,6 mg kg⁻¹**

Comparison	Diff of Means	t	P	P<0,05
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0,000 vs. 5,000	2,951	15,197	<0,001	Yes
0,000 vs. 2,500	2,438	12,556	<0,001	Yes

Comparisons for factor: **Dimethoate within 0%**

Comparison	Diff of Means	t	P	P<0,05
0,000 vs. 1,600	2,750	15,514	<0,001	Yes
0,000 vs. 0,800	2,043	11,525	<0,001	Yes
0,000 vs. 0,200	0,0731	0,413	0,968	No
0,000 vs. 0,100	0,0811	0,362	0,921	No
0,000 vs. 0,400	0,00232	0,0131	0,990	No

Comparisons for factor: **Dimethoate within 2,5%**

Comparison	Diff of Means	t	P	P<0,05
0,000 vs. 1,600	0,356	2,007	0,226	No
0,000 vs. 0,200	0,0941	0,531	0,974	No
0,000 vs. 0,400	0,0918	0,518	0,939	No
0,000 vs. 0,800	0,0779	0,439	0,886	No
0,000 vs. 0,100	0,0373	0,167	0,868	No

Comparisons for factor: **Dimethoate within 5%**

Comparison	Diff of Means	t	P	P<0,05
0,000 vs. 1,600	0,269	1,463	0,555	No
0,000 vs. 0,800	0,218	1,182	0,671	No
0,000 vs. 0,400	0,186	1,010	0,682	No
0,000 vs. 0,100	0,215	0,935	0,582	No
0,000 vs. 0,200	0,109	0,593	0,556	No

2. *Brassica rapa* germination test

a. Endpoint: Shoot length

Two Way Analysis of Variance

Comparisons for factor: **biochar within 0**

Comparison	Diff of Means	t	P	P<0,05
0,000 vs. 5,000	0,497	1,767	0,149	No
0,000 vs. 2,500	0,370	1,324	0,186	No

Comparisons for factor: **biochar within 10**

Comparison	Diff of Means	t	P	P<0,05
0,000 vs. 2,500	1,178	4,046	<0,001	Yes
0,000 vs. 5,000	0,844	2,936	0,003	Yes

Comparisons for factor: **biochar within 20**

Comparison	Diff of Means	t	P	P<0,05
0,000 vs. 5,000	0,975	3,359	0,002	Yes
0,000 vs. 2,500	0,0892	0,306	0,760	No

Comparisons for factor: **biochar within 30**

Comparison	Diff of Means	t	P	P<0,05
0,000 vs. 5,000	1,288	4,424	<0,001	Yes
0,000 vs. 2,500	0,611	2,096	0,036	Yes

Comparisons for factor: **biochar within 40**

Comparison	Diff of Means	t	P	P<0,05
0,000 vs. 5,000	0,769	2,561	0,021	Yes
0,000 vs. 2,500	0,194	0,662	0,508	No

Comparisons for factor: **biochar within 50**

Comparison	Diff of Means	t	P	P<0,05
0,000 vs. 5,000	0,899	3,073	0,004	Yes
0,000 vs. 2,500	0,342	1,077	0,282	No

Comparisons for factor: **dimethoate within 0**

Comparison	Diff of Means	t	P	P<0,05
0,000 vs. 50,000	3,342	11,353	<0,001	Yes
0,000 vs. 40,000	3,129	10,887	<0,001	Yes
0,000 vs. 30,000	3,041	10,578	<0,001	Yes
0,000 vs. 20,000	2,279	7,806	<0,001	Yes
0,000 vs. 10,000	1,483	5,161	<0,001	Yes

Comparisons for factor: **dimethoate within 2,5**

Comparison	Diff of Means	t	P	P<0,05
0,000 vs. 40,000	3,305	11,570	<0,001	Yes
0,000 vs. 50,000	3,370	11,090	<0,001	Yes
0,000 vs. 30,000	2,800	9,875	<0,001	Yes
0,000 vs. 20,000	2,560	9,153	<0,001	Yes
0,000 vs. 10,000	0,675	2,381	0,018	Yes

Comparisons for factor: **dimethoate within 5**

Comparison	Diff of Means	t	P	P<0,05
0,000 vs. 50,000	2,940	10,513	<0,001	Yes
0,000 vs. 40,000	2,858	9,709	<0,001	Yes
0,000 vs. 30,000	2,250	7,884	<0,001	Yes
0,000 vs. 20,000	1,801	6,442	<0,001	Yes
0,000 vs. 10,000	1,137	4,039	<0,001	Yes

b. Endpoint: Fresh weight

Two Way Analysis of Variance

Comparisons for factor: **biochar within 0**

Comparison	Diff of Means	t	P	P<0,05
0,000 vs. 5,000	0,669	1,203	0,406	No
0,000 vs. 2,500	0,134	0,242	0,809	No

Comparisons for factor: **biochar within 10**

Comparison	Diff of Means	t	P	P<0,05
0,000 vs. 2,500	1,810	3,145	0,003	Yes
0,000 vs. 5,000	0,950	1,673	0,095	No

Comparisons for factor: **biochar within 20**

Comparison	Diff of Means	t	P	P<0,05
0,000 vs. 5,000	0,724	1,263	0,371	No
0,000 vs. 2,500	0,344	0,596	0,551	No

Comparisons for factor: **biochar within 30**

Comparison	Diff of Means	t	P	P<0,05
0,000 vs. 5,000	2,239	3,891	<0,001	Yes
0,000 vs. 2,500	1,024	1,779	0,076	No

Comparisons for factor: **biochar within 40**

Comparison	Diff of Means	t	P	P<0,05
0,000 vs. 5,000	0,958	1,615	0,202	No
0,000 vs. 2,500	0,179	0,310	0,757	No

Comparisons for factor: **biochar within 50**

Comparison	Diff of Means	t	P	P<0,05
0,000 vs. 5,000	1,269	2,195	0,056	No
0,000 vs. 2,500	0,645	1,029	0,304	No

Comparisons for factor: **dimethoate within 0**

Comparison	Diff of Means	t	P	P<0,05
0,000 vs. 50,000	7,248	12,460	<0,001	Yes
0,000 vs. 30,000	6,883	12,119	<0,001	Yes
0,000 vs. 40,000	6,634	11,680	<0,001	Yes
0,000 vs. 20,000	5,082	8,809	<0,001	Yes
0,000 vs. 10,000	3,623	6,379	<0,001	Yes

Comparisons for factor: **dimethoate within 2,5**

Comparison	Diff of Means	t	P	P<0,05
0,000 vs. 40,000	6,321	11,198	<0,001	Yes
0,000 vs. 50,000	6,469	10,772	<0,001	Yes
0,000 vs. 30,000	5,726	10,219	<0,001	Yes

0,000 vs. 20,000	5,292	9,576	<0,001	Yes
0,000 vs. 10,000	1,680	2,998	0,003	Yes

Comparisons for factor: **dimethoate within 5**

Comparison	Diff of Means	t	P	P<0,05
0,000 vs. 50,000	5,309	9,607	<0,001	Yes
0,000 vs. 40,000	5,008	8,609	<0,001	Yes
0,000 vs. 30,000	3,975	7,049	<0,001	Yes
0,000 vs. 20,000	3,688	6,674	<0,001	Yes
0,000 vs. 10,000	2,004	3,603	<0,001	Yes

Table D. Information about the length variables' results for the calculus of Chi-square X^2 .

Variables	B0 test vs B25 test	B0 test vs B5 test	B25 test vs B5 test
Ymax1	5.17	5.17	5.62
Ymax2	5.62	5.66	5.66
EC ₅₀ 1	24.43	24.43	28.72
EC ₅₀ 2	28.72	44.04	44.04
b1	0.97	0.97	1.27
b2	1.27	0.95	0.95
Residue 1	644.73	636.60	641.836
Residue 2	646.65	652.40	656.105
<i>N</i>	420	430	434
EC ₅₀ mean	26.58	34.24	36.38
X^2	1.25	13.25	9.54

Table E. Information about the fresh weight variables' results for the calculus of Chi-square X^2 .

Variables	B0 test vs B25 test	B0 test vs B5 test	B25 test vs B5 test
Ymax1	139.47	139.47	140.88
Ymax2	140.88	123.79	123.79
EC ₅₀ 1	8.12	8.12	14.33
EC ₅₀ 2	14.33	17.07	17.07
b1	0.96	0.96	1.57
b2	1.57	0.99	0.99
Residue 1	911768.37	763418.40	991655.40
Residue 2	922808.87	774494.84	992862.43
<i>n</i>	420	430	434
EC ₅₀ mean	11.23	12.60	15.70
X^2	5.06	6.19	0.53